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Research Article

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Diabetes Type 1 Negatively Influences Leydig Cell Function in Rats, Which is Partially Reversible By Insulin Treatment

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Abbreviations: BB/OKL, Bio Breeding/Ottawa Karlsburg Leipzig rats; ELISA, enzyme-linked immunosorbent assay; FSH, follicle-stimulating hormone; HbA_{1c}, glycated hemoglobin A_{1c}; HC, healthy control; HRP, horseradish peroxidase; Insl3, insulin-like factor 3; LH, luteinizing hormone; MS, mass spectrometry; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, room temperature; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TBS, Tris-buffered saline; TNF, tumor necrosis factor α; TUNEL, terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling

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Abstract

Type 1 diabetes mellitus (T1DM) is associated with impaired spermatogenesis and lower testosterone levels and epididymal weight. However, the underlying processes in the testis are unknown and remain to be elucidated. Therefore, the present study focused on the effects of T1DM on testicular function in a spontaneously diabetic rat model. BB/OKL rats after diabetes manifestation were divided into 3 groups: those without insulin treatment and insulin treatment for a duration of 2 and of 6 weeks. Anthropometrical data, circulating levels of gonadotrophins, testosterone, and inhibin B were measured. Intratesticular testosterone, oxidative stress, inflammation, and apoptosis were analyzed. Key enzymes of steroidogenesis were evaluated in the testis. Untreated diabetic rats had significantly lower serum follicle-stimulating hormone and luteinizing hormone levels. Serum and intratesticular testosterone levels significantly decreased in untreated diabetic rats compared to healthy controls. Key markers of Leydig cell function were significantly

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downregulated at the RNA level: insulin-like factor 3 (*Insl3*) by 53% ($P = .006$), *Star* by 51% ($P = .004$), *Cyp11A1* by 80% ($P = .003$), *3Beta-Hsd2* by 61% ($P = .005$), and *Pbr* by 52% ($P = .002$). In the insulin-treated group, only *Cyp11A1* and *3Beta-Hsd2* transcripts were significantly lower. Interestingly, the long-term insulin–treated group showed significant upregulation of most steroidogenic enzymes without affecting testosterone levels. Tumor necrosis factor α and apoptosis were significantly increased in the long-term insulin–treated rats. In conclusion T1DM, with a severe lack of insulin, has an adverse action on Leydig cell function. This is partially reversible with well-compensated blood glucose control. Long-term T1DM adversely affects Leydig cell function because of the process of inflammation and apoptosis.

Key Words: fertility in diabetic rats, Leydig cell function, dysfunction of steroidogenesis

Insulin-dependent type 1 diabetes mellitus or type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by a lack of insulin-producing pancreatic β cells (1). According to the International Diabetes Federation, approximately 463 million adults were living with T1DM and type 2 (T2DM) in 2019; and it estimates that by 2045 this amount will increase to 700 million (2). More than 1.1 million children and adolescents are living with T1DM worldwide (2), and there is a continuous increase in the number of young patients with T1DM and T2DM (3, 4). The increased incidence of T1DM has been associated with falling birth rates and compromised fertility (5). Insulin deficiency and hyperglycemia lead to different effects adversely affecting the entire metabolism. Late diagnosed or poorly controlled T1DM results in cardiovascular disease, diabetic neuropathy, and retinopathy (6, 7). In the 11th century physicians already noticed that men with dysregulated glucose metabolism showed a "collapse of sexual functions" (8), but even well-adjusted patients frequently show end organ damage (8). Studies have shown that T1DM can lead to defects in testicular function, including spermatogenesis (9). Navarro-Casada et al have reported that rats with insulin deficiency and hyperglycemia induced by streptozotocin (STZ) showed teratozoospermia associated with low levels of serum testosterone and reduced epididymal weight (10, 11). A number of studies in men and in animal models of diabetes have reported alterations in spermatogenesis, degenerative and apoptotic changes in the testis, altered glucose metabolism in Sertoli cells, reduced testosterone synthesis and secretion, as well as ejaculatory dysfunction and reduced libido (9, 12-21). In a human study T1DM altered epididymal voiding, causing low ejaculate volume and mitochondrial damage resulting in decreased sperm motility (22). Hence fertility and sexuality are affected. However, only a limited number of studies have focused on the underlying mechanisms behind these observations. It has been suggested that endocrine disturbances, neuropathy, and increased oxidative stress could be mechanisms involved in the testicular and sexual dysfunction (17). Ballester et al assumed that Leydig cell function is negatively affected by the absence of insulin. This could also be due to an altered gonadotrophin balance with decreased follicle-stimulating hormone (FSH) production, which negatively affects spermatogenesis and fertility (10). However, the exact adverse effects of T1DM on testicular function remain unclear.

Insulin replacement therapy in diabetic rats may partly restore sperm counts and motility, as well as testosterone levels (23, 24). Schoeller et al suggested that insulin itself has no direct effect on testicular function but can restore fertility by supporting the functioning of the hypothalamo-pituitary-gonadal axis and therefore act via a central mechanism (9).

Published human studies have the limitation that for ethical reasons, nearly no testicular biopsies were available, therefore no systematic histopathology or molecular data from human testis of patients with T1DM are available. Many animal studies have employed STZ-induced diabetes. STZ is a toxic substance with actions very different from the autoimmune mechanisms involved in T1DM in humans, and thus employs a totally different pathogenicity. Therefore, we used spontaneously diabetic Bio Breeding/Ottawa Karlsburg Leipzig rats (BB/OKL) rats, a rat strain that represents a close homology to T1DM in humans. Like the human form, the disease has an early age of onset, occurs in lean animals, and is characterized by glucosuria, hyperglycemia, hypoinsulinemia, and ketoacidosis. BB/OKL diabetes is characterized morphologically by a β-cell–specific mononuclear cell infiltrate (insulitis) within the pancreatic islets of Langerhans. The autoimmune attack selectively destroys the insulin-producing β cells (25, 26). The aim of the present project was to investigate the effects of T1DM in BB/OKL rats without treatment and with short-term and long-term insulin replacement treatment on testicular function.

Material and Methods

Animal Model

Animal studies were approved by the local authorities of the state of Saxony, Germany, as recommended by the responsible local animal ethics review board (Landesdirektion Leipzig, T01/13, TVV60/13, TVV27/14, TVV59/15, Germany). BB/OttawaKarlsburg (F60/F99) strain were obtained from Laboratory Animal Science in Karlsburg at the University of Greifswald. BB/OK were kept and inbred as BB/OKL strain (F99/F12). BB/OKL rats were housed in pathogen-free facilities in groups of 3 in Macrolon type III (Ehret GmbH) at 22 ± 2 °C on a 12-hour light/dark cycle. Animals were fed a standard chow diet (Sniff) and had ad libitum access to water and food at all times. Glucose levels in rats were monitored routinely from age 6 weeks, twice a week or if the rats developed symptoms (including polyuria, polydipsia, change of fur, and weakness). Diabetes manifestation was defined as having measured 3 random elevated blood glucose levels (> 11.1 mmol/L) and increased glycated hemoglobin A_{1c} (HbA_{1c}) levels above normal range (> 5%) (27). After manifestation rats were separated into either a group without insulin treatment (NT = no insulin treatment) or an insulin replacement group (SIT = short-term insulin treatment). The NT group was humanely killed between day 7 and 14 after manifestation, depending on the health status of each individual rat, with sevoflurane anesthesia. The treatment groups received 1 to 2 sustained release insulin implants (Linplants, from LinShin) that were placed underneath the skin according to the company's protocol. Glucose levels were checked to be in the target range once a week. The first healthy, nondiabetic BB/OKL control group was also humanely killed after 14 days (HC = healthy control), and the second after 6 weeks (HCII = healthy control II).

Anthropometric Data

Before killing, the animals underwent an EchoMRI700 (Echo Medical Systems) analysis to obtain information about the amount of body fat, body fat content as percentage, and the lean mass of all animals, with 5 to 8 rats used in each group. A third group was treated for 6 weeks and had an age-matched HC group. Blood was collected directly from the heart and centrifuged at 1000*g* for 10 minutes to obtain serum. Testes were collected and testicular weight was measured. Serum samples, the testes, and other organs were frozen at –80 °C until they were used for hormonal measurements and steroidogenic genes analyses.

Hormone Levels Assay

Serum levels of rat luteinizing hormone (LH) and FSH were measured by specific enzyme-linked immunosorbent assay (ELISA; Cloud-Clone Inc) (28, 29). Intra-assay precision was coefficient of variation less than 10% and interassay precision was less than 12% for both gonadotropins. Serum and intratesticular levels of testosterone were quantified employing relevant ELISAs from DRG Diagnostics (30). Intra-assay and interassay coefficients for testosterone were 6.4% and 4%, respectively. Serum levels of inhibin B were analyzed by ELISA (Cloud-Clone Inc). Intra-assay and interassay coefficients for inhibin B were less than 10% and less than 12%, respectively.

Testicular Testosterone Analysis

Testosterone was extracted from testicular tissue as described previously (31). In brief, the tissue was weighed and homogenized by sonication (2 × 20 seconds) in a sodium phosphate-buffered saline (PBS) and then the homogenates centrifuged at 10 000*g* for 10 minutes. Testosterone was extracted with ethylacetate and evaporated overnight. The pellet was resuspended in PBS and intratesticular testosterone was assessed by an enzyme immunoassay kit (DRG Instruments GmbH) according to the manufacturer's instructions and by mass spectrometry (MS). For each sample the amount of testicular tissue was taken into consideration and the steroid concentration was measured per milligram of testicular tissue.

Mass spectrometry

To prove ELISA data, androgens, including testosterone, androstenedione, and DHT (5-dihydrotestosterone) were measured in serum and in testis tissue according to protocols established for human plasma analysis. All steroid hormones were determined by liquid chromatography–tandem MS, as previously described (32, 33). Briefly, sample aliquots, calibrators, and controls were combined with the internal standard mixture to monitor recovery. All samples were extracted using Oasis MAX SPE system plates (Waters). Chromatographic separation was carried out using a UPLC system connected to a Quattro Premier/XE triple Quad mass spectrometer (Waters). A Waters Acquity UPLC BEH C18 column (1.7 µm, 100 × 2.1) was used at a flow rate of 0.4 mL/min at 50 °C. Water and acetonitrile with 0.01% formic acid were used as the mobile phase. Two mass transitions were monitored for each hormone. Data were acquired using MassLynx 4.1 software and quantified with QuanLynx software (Waters).

During all analyses, the ambient temperature was maintained at 21 °C by air conditioning.

Assay to Evaluate Apoptosis

Fluorescein labels incorporated in nucleotide polymers were detected and quantified by fluorescence microscopy using the TUNEL (terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) assay from Roche. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. The kit was used according to the assay protocol from Roche. In brief, tissue sections were dewaxed and rehydrated according to standard protocols. Tissue sections were placed in 200-mL 0.1 M citrate buffer (pH 6.0.) and were irradiated in the microwave at 350 W for 5 minutes. Samples were rinsed with PBS twice and dried. Afterward, samples were incubated with TUNEL reaction mixture in a humidified atmosphere for 60 minutes at 37 °C in the dark. Slides were rinsed 3 times with PBS. After finishing all steps, samples were embedded with antifade and then analyzed under a fluorescence microscope using an excitation wavelength in the range of 450 to 500 nm and detection in the range of 515 to 565 nm. Calculation of TUNEL-positive cells was carried out blinded by counting 10 tubuli per slide in 5 animals per each group and the average was calculated.

Reactive Oxygen Species/Reactive Nitrogen Species Assay for Oxidative Stress

Oxidative stress was analyzed using the OxiSelect in vitro reactive oxygen species/reactive nitrogen species (ROS/RNS) assay kit. The OxiSelect in vitro ROS/RNS Assay Kit measures total ROS/RNS free radical activity. Samples are measured fluorometrically against a hydrogen peroxide or DCF (2′,7′-dichlorofluorescein) standard. The assay procedure was performed following the protocol instructions of the company. In brief, the tissue was weighed (30-60 mg/mL) and sonicated in PBS. The tissue sample and/or serum was centrifuged at 10 000*g* for 5 minutes. A 1:10 dilution series of DCF standards was performed and the relative fluorescence was evaluated with a fluorescence plate reader at 480 nm excitation/530 nm emission. In parallel, the hydrogen peroxide standard curve was prepared and measured. Samples are mixed and incubated for 5 minutes at room temperature (RT). Afterward, 100 µL of DCFH (2′,7′-dichlorodihydrofluorescein) solution was added to each well. The plate was protected from light and samples were incubated at RT for 15 to 45 minutes. After incubation, the fluorescence was measured at 480 nm excitation/530 nm emission. Data were evaluated taking the amount of testicular tissue into consideration.

Isolating RNA and Producing Complementary DNA

Total RNA was extracted from testis by RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. The RNA was pretreated with DNAse (RNase-free DNase Set, Qiagen) according to the manufacturer's instructions. The amount of total RNA was measured by Nanodrop (Thermo Fisher Scientific, NanoDrop2000 Spectrophotomer). The RNA was kept at –80 °C until further analyses were performed. Total RNA was further processed using iScript complementary (cDNA) Synthesis Kit (Bio-Rad Laboratories) as proposed in the manufacturer's protocol.

Gene Expression Analysis by Quantitative Polymerase Chain Reaction

The samples for quantitative polymerase chain reaction (qPCR) were prepared using iQSYBR Green Supermix (170-8882, Bio-Rad Laboratories) and the PCR cycles were run at 95 °C for 10 seconds, 56 to 62 °C for 45 seconds, 95 °C for 60 seconds, and 55 °C for 60 seconds followed by a melting curve from 55 to 95 °C in steps of 0.5 °C and then held at 4 °C (iCycler iQ, Bio-Rad Laboratories) after having estimated the best reaction conditions by running a temperature gradient. All values were normalized to β-actin for testicular tissue to balance possible irregularities in RNA concentration. To control effectivity of the process, negative control (RT-) was always added to each qPCR assay. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold changes in gene expression. Primer sequences are depicted in Supplementary Table 1 (34).

Automated Western Blot Analysis

Automated Western blotting of steroidogenic enzymes was performed as described earlier (35). Briefly, the testicular tissue was lysed in CellLytic cell reagent (Sigma Chemical Co) and the lysates diluted with sample buffer to a protein concentration of 0.3 µg in 4 µL were mixed together with the 5× Master Mix (DTT, fluorescence-labeled marker, sodium dodecyl sulfate) in a ratio of 5:1 and then incubated at 95 °C for 5 minutes. The samples, the biotin-labeled protein ladder, blocking reagent, primary antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies, chemiluminescent substrate, and stacking matricies were loaded into individual wells of the sample plate. Antibodies were diluted with antibody diluent buffer. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated in the Wes instrument. The 30-minute incubations

with primary antibodies against *Star* (dilution 1:100, Santa Cruz) (36), *Cyp11A1* (1:300, Abcam) (37), *Cyp17A1* (1:50, Abcam) (38), and *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase; dilution 1:150, Santa Cruz) (39) were followed by incubation with HRP-conjugated goat antirabbit secondary antibody for 30 minutes (34). Luminol and peroxide (ProteinSimple) were then added to generate chemiluminescence. The digital images obtained were analyzed with Compass software (ProteinSimple). Band densities were normalized against *Gapdh* and the ontogenetic expression of steroidogenic enzymes was demonstrated as percentages of the corresponding values of testicular tissue in healthy controls.

Immunohistochemical Staining for Leydig Cell Markers and Tumor Necrosis Factor α

The samples were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated with gradually increasing concentrations of aqueous ethanol, embedded in paraffin (P3808, Sigma Aldrich) and cut into 4- to 5-µm thick sections and placed on glass slides (J1800AMNZ, Gerhard Menzel) for morphological analysis and staining.

Samples for immunofluorescence staining were first subjected to antigen retrieval using citrate buffer (pH 6.0) in a water bath at 95 °C for 30 minutes and cooled down for another 30 minutes. Afterward, slides were incubated for 30 minutes in 10% donkey serum (017-000-001, Jackson ImmunoResearch) diluted in 1% bovine serum albumin-1 × Tris-buffered saline (TBS). To explore Leydig cell markers, we stained for *Star* and *Cyp17A1*. After incubation with a primary antimouse antibody (1:100, Santa Cruz) or antirabbit (1:50, Novus Biology) as well as unspecific immunoglobulin Gs (used as negative controls at the same concentration) dissolved in 1% bovine serum albumin–TBS overnight at 4 °C, the slides were washed 3 times for 5 minutes each with TBS. The specificity of tumor necrosis factor α (TNF-α) antibody was tested by preabsorption with antigen A. Antirabbit TNF-α antibody was incubated with 10-fold excess of recombinant rat TNF-α (Sigma Aldrich) (40) at 4 °C overnight (34).

Slides were then incubated either with a secondary antibody conjugated to Cy3 or with a secondary antibody conjugated to HRP depending on the type of staining. The secondary antibodies were diluted in blocking serum and incubated for 30 minutes at RT. After washing, the samples were incubated with TSA-Plus Fluorescein (NEL741001KT, PerkinElmer) or TSA-Plus Cy3 (NEL744001KT, PerkinElmer) according to the manufacturer's protocol. After washing, the samples were mounted in Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; H-1500, Vectro). All stained sections were photographed using a microscope (Eclipse E800; Nikon) with a 12.5-million pixel, cooled digital color camera (Olympus DP70). Calculation of TNFα-positive cells was carried out blinded by counting 10 tubuli per slide in 5 animals per each group and the average was calculated.

Morphology of Testicular Tissue

Testicular tissue was fixed in 4% paraformaldehyde and processed for paraffin-embedding and sectioning. In more detail, the morphology of the seminiferous tubuli was employed to assess the effect of diabetes with or without insulin replacement therapy on the spermatogenic process. The different spermatogenic stages were identified on the basis of cell types (defined by size, shape, and location in the seminiferous tubuli), according to Russell and colleagues (41). Ten randomly selected fields of hematoxylin-eosin–stained slices of testis were evaluated per animal. Fifty tubuli were analyzed per each slide at 20× magnification with a Nikon microscope.

Statistical Analysis

Differences in the values obtained for animals subjected to different treatments were evaluated for statistical significance with *t* test or one-way analysis of variance, in case of 2 or more than 2 groups, respectively. If the normality test failed, we used analysis of variance on ranks employing the SigmaStat (version 11.00) software package (SPSS Inc). Furthermore, the Dunn method was used (all pairwise multiple comparison procedures and multiple comparisons vs HC group). *P* less than .05 was considered statistically significant, and *P* less than .01 was classified as highly significant.

Results

Body Weight and Percentage of Body Fat Were Negatively Affected by Diabetes Manifestation

As expected, the body weight was significantly lower in the NT group by 33% (317.3 g ± 50.2, *P* = .002) and in the SIT group by 12% (384.3 g ± 34.3, *P* = .007) compared to HCs (430.4 g ± 8.2). The body weight of the long-term treated rats was 444.3 g ± 14.1 compared to their HCs (477.2 g ± 15.8, *P* = .014) (Table 1). In addition, the percentage of body fat, the body fat in grams, and the lean mass were significantly lower in the NT group (see Table 1). Testis weights were comparable between all experimental groups and ranged from 1.57 g ± 0.03 in the HC group to 1.47 g ± 0.35 in the NT group (see Table 1).

Table 1. Anthropometric data, metabolic parameters and gonadotrophin levels in male BB/OKL rats

Treatment groups	Body wt, g	*P*	Body fat, %	*P*	Body fat, g	*P*	Lean mass, g	*P*	Testis wt, g	*P*	HbA_{1c}, %	*P*	LH, ng/mL	*P*	FSH, ng/mL	*P*
HC	430.38 ± 8.2		8.51 ± 0.34		36.66 ± 1.5		374.78 ± 7.7		1.57 ± 0.03		4.23 ± 0.07		2.34 ± 1.03		43.24 ± 3.20	
NT	317.33 ± 50.2	.004	4.86 ± 0.97	.004	13.54 ± 3.1	< .001	265.69 ± 68.4	.003	1.47 ± 0.35	.43	6.67 ± 0.91	< .001	1.23 ± 1.3.6	.048	29.83 ± 10.9	.024
SIT	384.28 ± 34.3	.005	6.67 ± 0.89	.002	26.61 ± 2.8	< .001	325.84 ± 28.5	.007	1.68 ± 0.24	.59	5.04 ± 0.57	.030	3.05 ± 1.14	.26	42.54 ± 8.91	.027
HCII	477.18 ± 15.8		10.04 ± 1.30		48.02 ± 6.8		399.42 ± 12.3		1.74 ± 0.08		4.20 ± 0.06		4.73 ± 1.11		2.34 ± 1.03	
LIT	444.25 ± 14.1	.014	8.82 ± 1.31	.204	39.26 ± 6.5	.093	378.50 ± 12.3	.40	1.64 ± 0.24	.69	5.75 ± 0.73	.002	4.93 ± 1.01	.77	2.34 ± 1.03	.218

Anthropometric data and metabolic parameters in male BB/OKL rats. Results are represented as means. *P* values are given between HCs vs NT and NT vs SIT. N = 5 to 10 rats per group.
LH levels as well as FSH levels are significantly reduced in NT compared to HC animals. *T* test or one-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SD. *P* values are given between HCs vs NT and NT vs SIT.
Abbreviations: FSH, follicle-stimulating hormone; HbA_{1c} glycated hemoglobin A_{1c}; HC, healthy controls; HCII, healthy controls; IT, insulin treatment; LH, luteinizing hormone; LIT, long-term insulin treatment; NT, no insulin treatment; SIT, short-term insulin treatment. N = 5 to 8 rats per group.

Glycated Hemoglobin A_{1c} Increased in Diabetic Rats

As expected, HbA_{1c} levels were significantly higher in the SIT group by 19% (5.04%, *P* = .001) and in the NT group by 57% (6.67%, *P* < .0001) compared to HCs (4.23%). Rats on the longer insulin treatment also had significant higher HbA_{1c} levels by 37% (5.75%, *P* = .002) compared to their age-matched HCs (4.20%) (see Table 1).

Gonadotrophins Decreased in Untreated Diabetic Rats

LH levels were significantly lower in the NT group (by 50.9%, 1.2 ng/mL, *P* = .048) compared to HCs (2.34 ng/mL) and insulin-treated groups (by 71.2%, 3.1 ng/mL, *P* = .014). Serum FSH levels were significantly lower in the NT group by 31% (29.8 ng/mL, *P* = .024) and compared to the treated group by 29.9% (42.5 ng/mL, *P* = .027). Again there was no difference between the FSH levels of the HC (43.2 ng/mL) and the treated group (42.5 ng/mL) (see Table 1).

Serum and Intratesticular Testosterone Levels Significantly Decreased in Nontreated Diabetic Rats

NT diabetic rats had significantly lower serum and intratesticular testosterone levels, which were measured by ELISA. Our ELISA results were supported by MS data. We measured significantly lower serum testosterone levels by 64.4% (0.23 nmol/L, *P* = .009) compared to HCs and by 75.3% (0.65 nmol/L, *P* ≤ .001) compared to rats treated with insulin (0.93 nmol/L). Furthermore, intratesticular testosterone was also significantly lower in the NT group by 70.9% (26.37 nmol/L, *P* = .004) compared to HCs (90.8 nmol/L) and by 75.9% (109.7 nmol/L, *P* < .001) compared to SIT. Furthermore, intratesticular androstenedione was significantly higher in the SIT group (23.3 nmol/L) compared to the NT group (4.7 nmol/L, increase by 79.8%, *P* = .002). Intratesticular DHT was significantly reduced by 58.6% in the NT group (1.30 nmol/L) compared to the HCs (3.16 nmol/L) (Fig. 1A-1D).

Insulin-Like Factor 3, a Marker of Leydig Cell Function and Differentiation, Is Downregulated in Untreated Rats

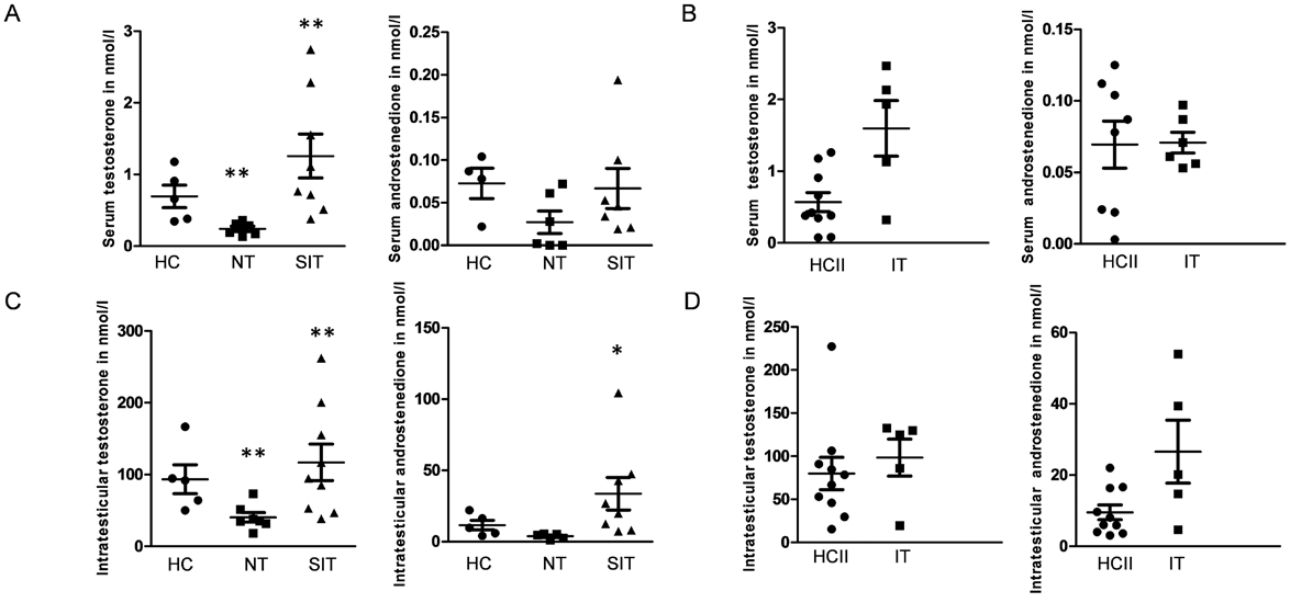
We measured insulin-like factor 3 (*Insl3*) expression in the testis at the transcriptional level. In our cohort we found a downregulation of *Insl3* by 53% (*P* = .006) in the NT

group compared to HCs. With insulin replacement therapy, the levels increased to higher expression levels than in the HCs (Fig. 2).

In the group that was treated with insulin for a longer duration, we saw an upregulation of *Insl3* by +172% (P = .01) compared to HCs.

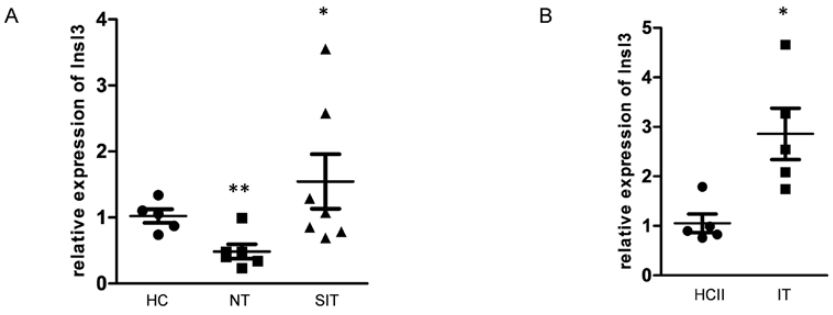
Steroidogenic Enzymes Were Significantly Downregulated in the Untreated Diabetic Rats, Which Was Partially Reversible With Insulin Treatment

Markers for Leydig cell function and steroidogenesis were significantly downregulated at the transcriptional level in



Serum and intratesticular testosterone and androstenedione in nmol/l
HC: healthy control, SIT= short term insulin treatment, NT= no treatment; HCII= healthy control; IT= insulin treatment;
One-way ANOVA was applied. All results are represented as means ± SEM; p<0.05=; p<0.01=***

Figure 1. A to D, Serum and intratesticular testosterone and androstenedione levels (A-D). A and B, Serum testosterone levels in nanomole per liter were significant reduced in nontreated (NT) compared to healthy control (HC) animals and a short-term insulin treated (SIT) group. C and D, Testosterone measurements in testis were calculated per milligram testis weight and are given in nanomole per liter per mg tissue and showed significant reduced levels in NT compared to HC rats. One-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SEM. **P* less than .05; ***P* less than .01. HCII, healthy control; IT, insulin treatment. N = 5 to 8 rats per group.



Insl3 mRNA expression in the testis
HC: healthy control, NT= no treatment, SIT= short term insulin treatment; HCII= healthy control; IT= insulin treatment;
One-way ANOVA was applied. All results are represented as means ± SEM; p<0.05=; p<0.01=***

Figure 2. Insulin-like factor 3 (*Insl3*) expression in the testis. Expression levels were calculated relative to the housekeeping gene β-actin and healthy control (HC) rats at the messenger RNA level. A, No-treatment (NT) reduced *Insl3* expression in testis compared to HC rats. B, Chronic long-term insulin treatment (IT) significantly elevated testis *Insl3* expression. One-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SEM. **P* less than .05; ***P* less than .01. HCII, healthy control; IT, insulin treatment; SIT, short-term insulin treatment. N = 5 to 8 rats per group.

the NT group: *Star* by 51% ($P = .004$), *Cyp11A1* by 80% ($P = .003$), *3Beta-Hsd2* by 61% ($P = .005$), and *Pbr* by 52% ($P = .002$). These data match the lower serum and intratesticular testosterone levels. Interestingly, only one steroidogenic gene was upregulated: *Cyp17A1* by +451% ($P = .002$). In the SIT group, only *Cyp11A1* and *3Beta-Hsd2* were significantly downregulated by 51% ($P = .019$) and 37% ($P = .03$) (Fig. 3A-3F). Thus, the downregulation for *Star*, *Pbr*, and *Insl3* was reversible with insulin treatment. Sertoli and germ cell markers and inhibin B levels were not affected.

Steroidogenic enzyme protein expression was also downregulated in the NT group compared to HCs: *Star* by 47.5% ($P \leq .001$) and *Cyp11A1* by 50.3% ($P = .011$) (Fig. 4A-4C, 4G). In the SIT group we still found a significant downregulation of *Cyp11A1* as at the transcript level by 36.7% ($P = .027$), whereas *Star* and *Cyp17A1* expression did not significantly change compared to the NT control (Fig. 4A-4C, 4G). Furthermore, with immunohistochemical staining we saw less staining for *Star* and an increased staining for *Cyp17A1* in the NT diabetic rats (Supplementary Fig. 2) (34).

Steroidogenic Enzymes Were Significantly Upregulated in the Long-Term Treatment Group

Interestingly, the long-term treatment group showed a significant upregulation for the following steroidogenic genes: *Star* (+133%, $P = .010$), *Cyp11A1* (+241%, $P < .001$), *Cyp17A1* (+858%, $P = .008$), *3Beta-Hsd2* (+137%, $P = .008$), *17Beta-Hsd3* (+61%, $P = .045$), *Pbr* (+171%, $P = .008$), and *Insl3* (+172%, $P = .01$) (Fig. 3G-3L). This did not result in a significant increase in testosterone levels. Again, Sertoli and germ cell markers (including *FSH receptor, Inhibin B, Vimentin*, *AMH*, *DDX4*, *CREM*, and *Acrosin*) and inhibin B levels were not affected in the long-term treatment group. At the protein level we did not find significant differences for *Star*, *Cyp11A1*, and *Cyp17A1* expression (Fig. 4D-4G).

Type 1 Diabetes Mellitus Disrupted Testis Morphology Resulting in a Reduced Number of Spermatids and an Increase in Morphologically Abnormal Tubuli

In the NT group we found elongated spermatids in only 83.3% of the tubuli compared to 100% in HCs ($P < .001$).

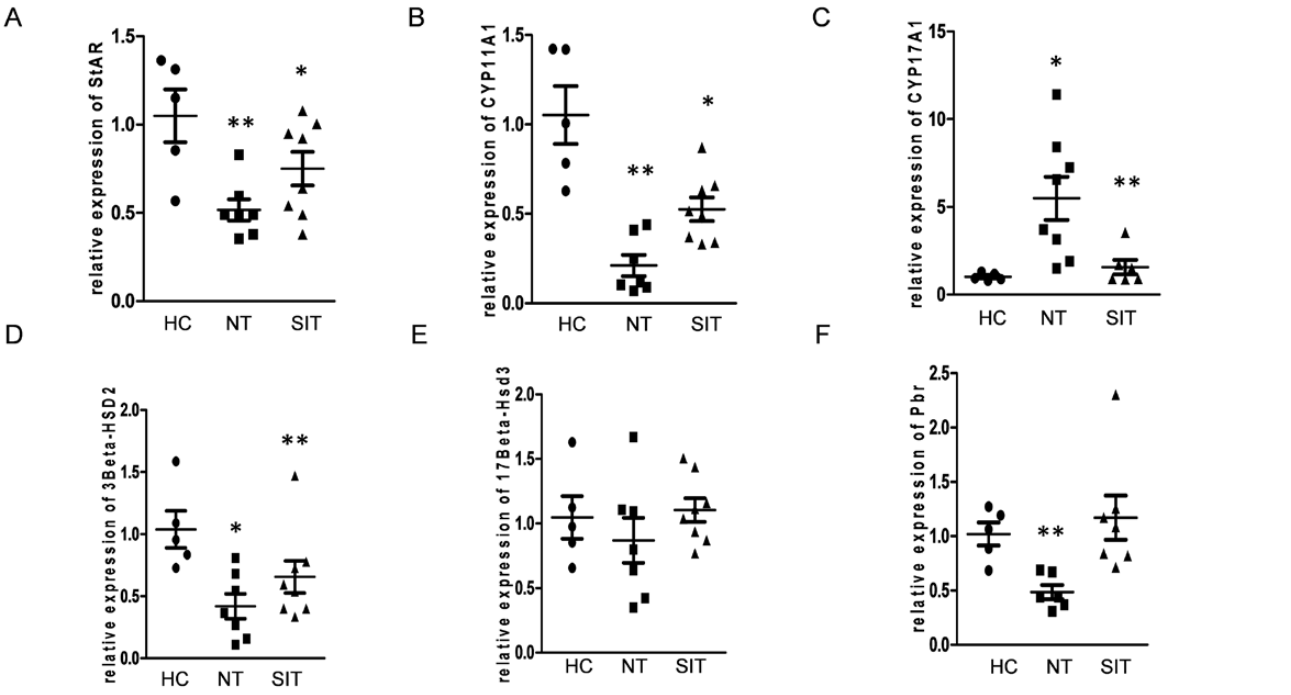


Figure 3. Steroidogenic gene expression in the testis. Decreased expression levels of steroidogenic enzymes were found in untreated (NT) rats: A, *Star*; B, *Cyp11A1*; C, *Cyp17A1*; D, *3Beta-Hsd2*; E, *17Beta-Hsd3*; and F, *Pbr*. Increased expression levels were found after long-term insulin treatment: G, *Star*; H, *Cyp11A1*; I, *Cyp17A1*; J, *3Beta-Hsd2*; K, *17Beta-Hsd3*; and L, *Pbr*. Expression levels were calculated relative to housekeeping gene β-*actin* and healthy control (HC) rats. One-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SEM. **P* less than .05; ***P* less than .01; and always compared to HC. HCII, healthy control; IT, insulin treatment; SIT, short-term insulin treatment. N = 5 to 8 rats per group.

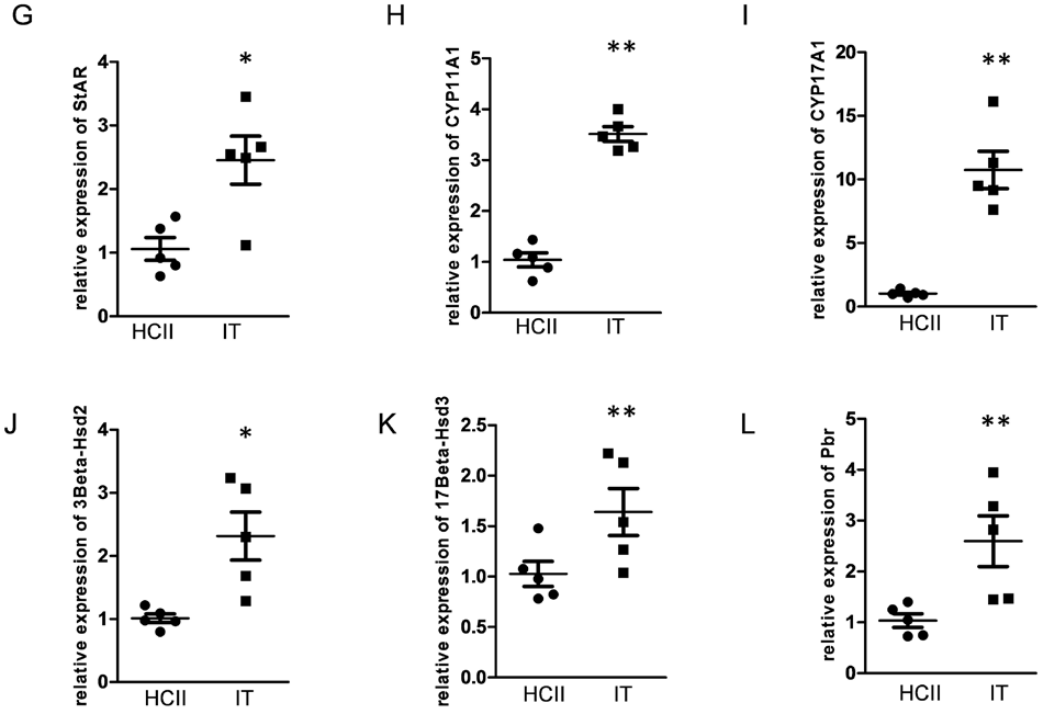
Furthermore, round spermatids were found in 56% of the tubuli of NT rats compared to 70% in the HC group ($P = .006$). The prevalence of abnormal tubuli with no normal structure increased from 8% to 20.6% in the untreated group ($P = .002$).

In the SIT group we again found significantly less-elongated spermatids (–10.5%, $P < .001$) and fewer tubuli in stages VII to XII (by –21%, $P = .002$). Again there were 20% abnormal tubuli compared to 8% in the HC group ($P = .002$).

In the long-term treatment group we found significant differences for the amount of elongated spermatids (–5.7%, $P = .002$) and for tubuli only in stages VII to XII (–9.9%, $P < .001$) (Supplementary Fig. 1) (34).

Total Reactive Oxygen Species/Reactive Nitrogen Species Activity Was Higher in the Serum of Untreated Rats

The total ROS/RNS free radical activity, a marker for oxidative stress, was only increased in the serum of NT rats by 280% ($P = .047$) compared to HCs and by 304% ($P < .001$) in the treated group. There was no difference between the HC group and the insulin treated group for ROS/RNS activity in serum. Additionally, we measured ROS/RNS activity in the testicular tissue, which did not show significant differences in the NT or SIT group. In the long-term treatment group, there was an increase of ROS/RNS activity by 83%, but this was not significant because of great interindividual variation (Fig. 5B).



Relative expression of steroidogenic enzymes in the testis (G-L)
HC: healthy control, SIT= short term insulin treatment, NT= no treatment; HCII= healthy control; IT= insulin treatment; One-way ANOVA was applied. All results are represented as means ± SEM; p<0.05=; p<0.01=***

Figure 3. Continued.

Fewer Apoptotic Cells and Lower Tumor Necrosis Factor α Levels After Diabetes Manifestation

After diabetes manifestation, we found significantly fewer apoptotic cells in the testis compared to HCs: by 38.8% ($P = .029$) in the NT group and even by 62.6% ($P = .03$) in the SIT group (Fig. 5A).

Shortly after manifestation, there was no inflammation found in the testicular tissue. Unexpectedly, the intratesticular TNF-α expression even decreased by 40.2% ($P = .007$) in the NT group compared to HCs. There was no significant statistical difference compared to the SIT group (Fig. 5C and 5D).

Increased Apoptosis and Inflammation in the Long-Term Treatment Group

After insulin treatment for 6 weeks, we found increased TNF-α expression and more apoptotic cells in the testis.

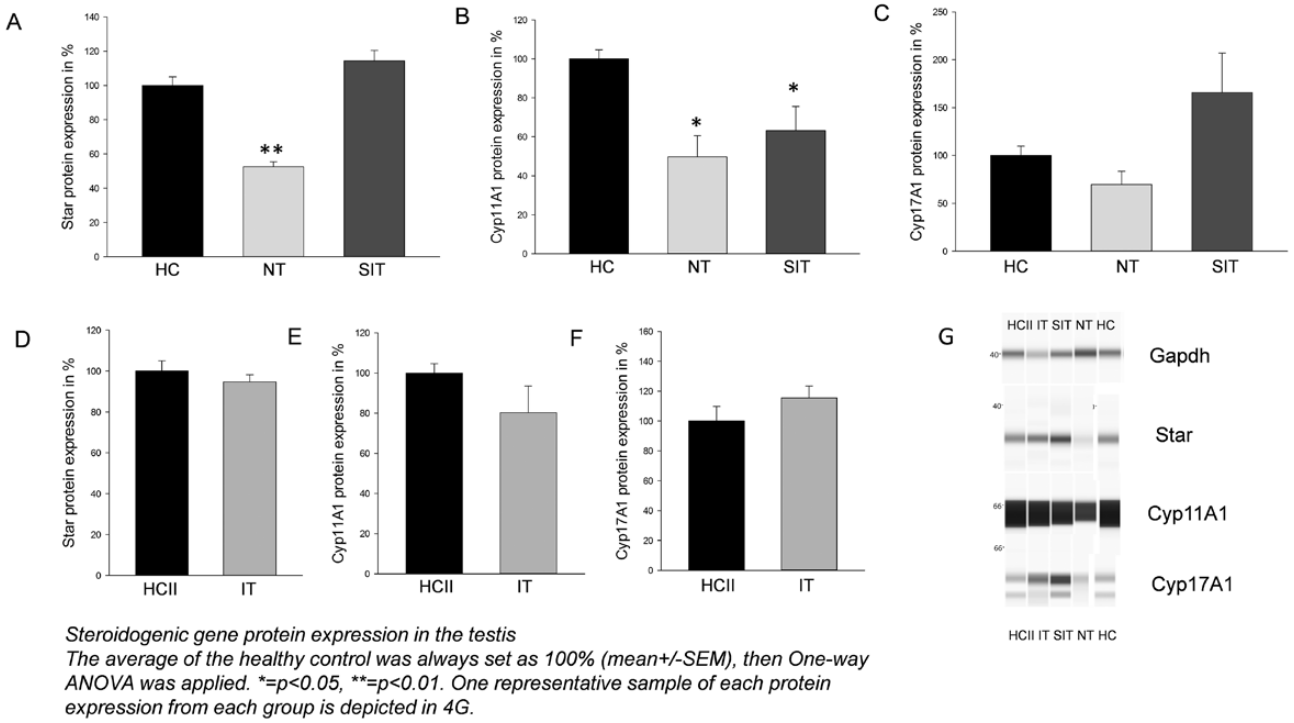


Figure 4. Steroidogenic gene protein expression in the testis. Decreased expression levels of steroidogenic enzymes on the protein level were found in untreated (NT) rats: A, *Star*; B, *Cyp11A1*; and C, *Cyp17A1*. No significant differences were found for expression levels after long-term insulin treatment: D, *Star*; E, *Cyp11A1*; and F, *Cyp17A1*. Protein expression of steroidogenic enzymes in testicular tissue in relation to Gapdh was evaluated. One-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SEM. **P* less than .05; ***P* less than .01. One representative sample of each protein expression from each group is depicted in G. Gapdh, glyceraldehyde 3-phosphate dehydrogenase; HC, healthy control; HCII, healthy control; IT, insulin treatment; SIT, short-term insulin treatment. N = 5 to 8 rats per group.

TNF-α expression in the testis increased in the long-term treatment group by +148.1% ($P \leq .001$). Furthermore, we used a TUNEL assay and counted the amount of apoptotic cells. As expected we found an increase in apoptotic cells by +264% ($P = .005$) in the long-term treatment group, although the rats had relatively unaffected HbA_{1c} levels. Therefore, after a longer manifestation of diabetes we found signs of inflammation, even under good blood sugar control (Fig. 5A, 5C-5E).

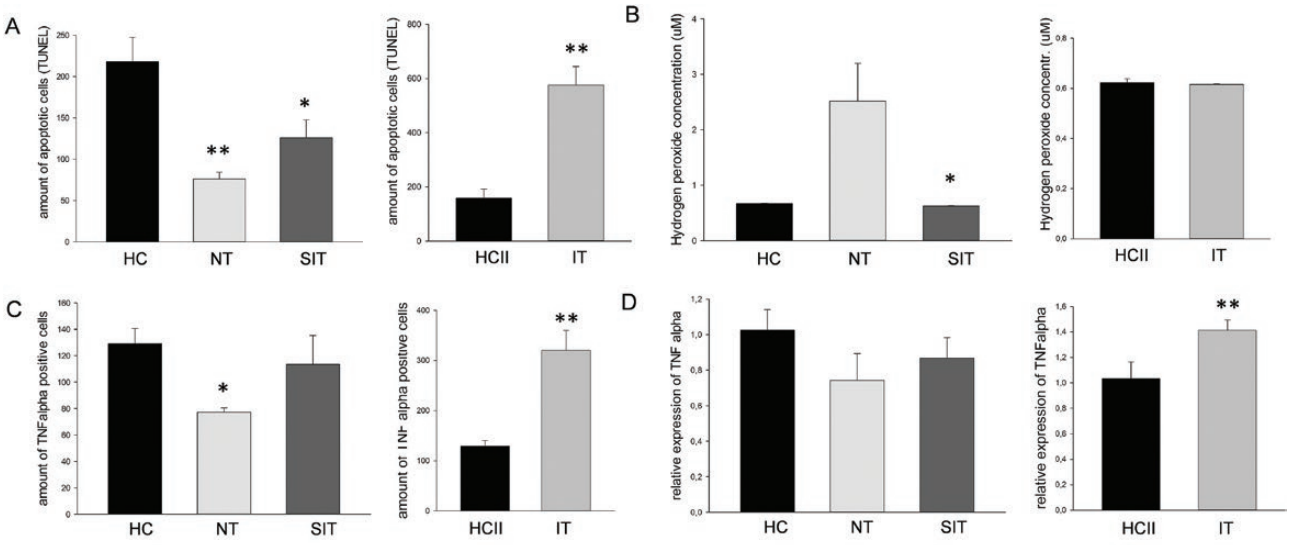
Discussion

Acute manifestation of diabetes, without insulin treatment, negatively affects Leydig cell function. Steroidogenic enzymes, as assessed at transcript and protein levels, were significantly downregulated, which were associated with significantly lower levels of serum and intratesticular testosterone. The feedback mechanisms did not adequately increase gonadotrophin levels, pointing toward an additional central defect. Directly after manifestation, there was no increase in inflammatory markers and fewer apoptotic cells but signs of higher levels of oxidative stress. We suggest that either the lack of insulin or the hyperglycemia after acute manifestation negatively affects testicular function without inducing inflammation.

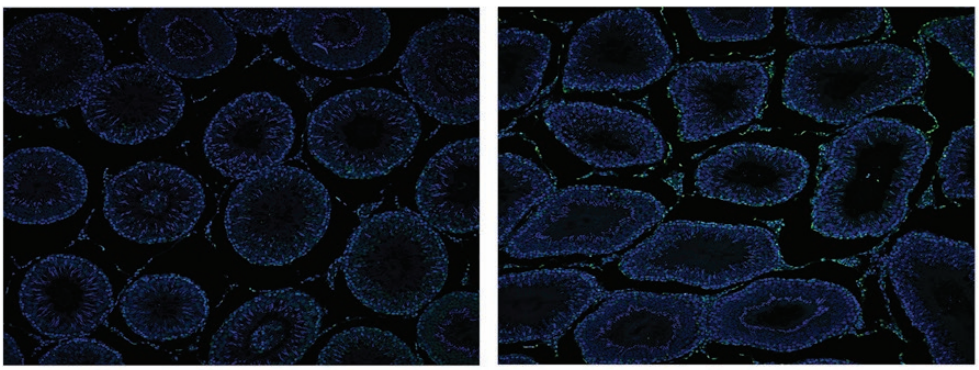
As expected, the body weight of the rats was significantly lower in the NT group and in the SIT group compared to HCs. The testicular weight was lower in the NT group but the differences were not significant. Other authors have demonstrated that diabetes decreased the weight of the testes, seminal vesicle, and epididymis in STZ-induced diabetic rats, whereas insulin treatment restored these organs' weights (42-46).

There is a wide discrepancy concerning the reported effects of diabetes and insulin treatment on gonadotrophin levels, ranging from a total recovery of LH (46-48) and FSH (45, 47) to a lack of recovery of LH (45) and FSH (46, 48). In the present study LH and FSH levels were significantly lower in the NT diabetic group compared to HCs and compared to the treated group. Insulin replacement therapy resulted in recovery of LH and FSH levels, thus implying that the central regulatory defect is reversible with insulin. This is line with the hypothesis of Schoeller et al that low insulin levels lead to decreased leptin levels, leading to less gonadotropin-releasing hormone secretion and subsequently lower LH and FSH signaling to the testis (9).

Circulating testosterone levels usually show a strong variation in rats. Other investigators were also able to show decreased serum testosterone concentrations in diabetic rats (43, 46, 49-52) and in humans with diabetes (53-56).



Possible pathomechanisms: Apoptosis, oxidative stress and inflammation
A: TUNEL assay, depicted are the amount of apoptotic cells per ten tubuli/testis
B: Hydrogen peroxide concentration in µM as a paramater of ROS/oxidative stress
C: staining positive cells for TNF-alpha, always counted in ten tubuli/testis per slide
D: relative expression of TNF-alpha on RNA level, always compared to healthy control
HC: healthy control, NT= no treatment, SIT= short term insulin treatment ; HCII= healthy control; IT= insulin treatment;
One-way ANOVA was applied. All results are represented as means ± SEM; p<0.05=; p<0.01=***



TUNEL assay, depicted are the apoptotic cells stained in green colour; HCII= healthy control; IT= insulin treatment

Figure 5. Apoptosis, oxidative stress and inflammation in the testis. A, TUNEL assay: Apoptotic cells decreased after diabetes manifestation and increased after long-term insulin treatment. Depicted is the number of apoptotic cells per 10 testis. B, Oxidative stress: Oxidative stress was increased in serum after diabetes manifestation. Hydrogen peroxide concentration in micromolars (µM) as a parameter of reactive oxygen species/oxidative stress. C and D, Inflammation: Inflammation was only significantly increased after long-term insulin treatment. Cells staining positive for Tnf-α were always counted as 10 testis per slide. D, Relative expression of *Tnf-α*, always compared to healthy controls (HCs). E, TUNEL assay, depicted are the apoptotic cells stained in green (magnification 1 × 40). One-way analysis of variance was applied for statistical evaluation. All results are represented as means ± SEM; **P* less than .05; ***P* less than .01. HC, healthy control; HCII, healthy control; IT, insulin treatment; NT, no treatment; SIT, short-term insulin treatment; Tnf-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidal transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling.

BB/OKL diabetic rats had significantly lower serum testosterone levels compared to HCs and compared to rats treated with insulin. There is just one other study published that also examined intratesticular testosterone levels, and they also found reduced intratesticular testosterone levels (52). We also measured significantly lower intratesticular testosterone in the NT group compared to HCs and compared to SIT. It is unclear why lower testosterone levels do not result in increased gonadotrophins via the negative feedback axis affecting the hypothalamus and the pituitary. Ballester et al (10) and Schoeller et al (9) suggested that the Leydig cell function is negatively affected by the lack of stimulatory effects by insulin. They describe that insulin deficiency leads to a decreased gonadotropin-releasing hormone release, which results in lower gonadotrophin levels, which negatively affects spermatogenesis and fertility. This

fits our data that after some time of insulin treatment, gonadotrophin and testosterone levels normalize again. Another possible mechanism was published by Rato et al: Sertoli cells that were exposed to T2DM conditions exhibited intracellular glycogen accumulation. Progressive stages of diabetes mellitus lead to gradually reduced levels of testosterone that favor metabolic reprogramming toward glycogen synthesis (57). A similar mechanism could be a reason for decreasing testosterone in patients with T1DM.

There are no studies published in humans and only a very few in diabetic rats that have analyzed Leydig cell function. Adewole et al and Ballester et al found a reduced number of Leydig cells (10, 27) and Kianifard et al and Trindade et al showed the presence of lipid droplets in Leydig cells (58, 59). Ermetici et al analyzed patient data and found significantly lower *Insl3* concentrations in serum, suggesting an early impairment of Leydig cell function (55). To our knowledge, the present work is the first to analyze the function and steroidogenesis of Leydig cells in diabetic rats and especially in testicular tissue. *Insl3* is produced and secreted by Leydig cells in the testes and reflects the differentiation status and number of Leydig cells. It provides a reliable assessment of Leydig cell differentiation (60) and is not regulated by the hormones of the hypothalamic-pituitary-gonadal axis. In our cohort we found a downregulation of *Insl3* transcripts in the untreated group. Therefore, Leydig cell function and differentiation might be negatively affected after diabetes manifestation. Insulin replacement therapy might successfully restore *Insl3* transcript levels. Therefore, we speculate that insulin is important for normal primary Leydig cell function and differentiation.

Expression levels of steroidogenic enzymes were significantly downregulated at the messenger RNA level in the NT group, including *Star*, *Cyp11A1*, *3Beta-Hsd2*, and *Pbr*. These findings may explain the lower intratesticular and serum testosterone levels observed in NT diabetic rats because of decreased androgenic steroidogenesis in Leydig cells. Interestingly, *Cyp17A1* was significantly upregulated at the transcript level and at the protein level measured with immunohistochemical staining but not with Western blot analysis. Similarly, increased expression of this steroidogenic enzyme was found in the ovaries of women with polycystic ovary syndrome and was associated with diabetes (61). The reason remains unclear but there is evidence that *Cyp17A1* expression can be regulated by insulin (61). Although upregulation of this one gene cannot compensate for or improve testosterone production, especially because this was found at the transcriptional level only and not at the protein level, the relevance remains unclear.

In diabetic rats treated with insulin, gene expression of *Pbr* and *17Beta-Hsd3* was restored to the expression levels of HCs. The expression of *Cyp11A1* and *3Beta-Hsd2* was still significantly downregulated compared to HCs but already significantly upregulated compared to NT controls. Hence, we can conclude that Leydig cell function and especially steroidogenic enzyme expression might be negatively influenced by changes in the endocrine and paracrine milieu caused by T1DM. The lack of insulin or the hyperglycemia may play an important role. Alves et al suggest that testicular cells have their own glucose-sensing machinery that reacts to hormonal fluctuations and have several mechanisms to counteract hyperglycemic and hypoglycemic events (62). The metabolic cooperation between testicular cells is crucial for normal spermatogenesis. Sertoli cells are responsible for the physical support of germ cells and for lactate production (62). Our study shows that if insulin treatment is started, the negative effects on Leydig cell steroidogenesis are partially reversible.

Rats that were treated with insulin replacement for longer than 6 weeks unexpectedly showed a significant upregulation of *Insl3* and all steroidogenic enzymes. This effect, however, was not associated with significant changes in serum and intratesticular testosterone concentrations. We have seen a similar effect in a study in which we analyzed the effects of childhood obesity on testicular function. We also found an upregulation of the steroidogenic enzymes after a short time of obesity and after the process of inflammation has started. We speculated that this might be a compensatory mechanism of the body trying to keep testosterone levels in the normal range (63). At some point this mechanism might fail and cannot compensate anymore, hence steroidogenic enzymes expression might be reduced and serum and intratesticular testosterone levels might decrease.

Other groups have found negative effects of diabetes on germ cells. Murray et al showed severe germ cell depletion (49), Cai et al found a reduction of the seminiferous tubule diameter and an increase of degenerating germ cells (42) and Alves et al showed negative effects in germ cell function in testicular biopsies from diabetic men. Decreased levels of lactate, alanine, citrate, and creatine were measured and the expression of glucose transporter 1 and 3 and phosphofructokinase 1 were decreased in testicular biopsies, thereby influencing germ cell development and support (64). However, most animal studies do not report any effects on germ cell function and morphology. In most cases it remains unclear whether germ cell effects were not evaluated in the studies or if data are not presented. The outcome of normal germ cell function is of course the basis of spermatogenesis and undisturbed sperm function. In STZ-induced diabetic mice and rats, the sperm count and motility decreased and there was an increase in sperm DNA fragmentation and denaturation (43, 65), and teratozoospermia was observed

(11). Most studies showed negative effects on sperm morphology and motility and also an increased sperm DNA fragmentation index (12, 66, 67).

Unfortunately, we did not have the possibility to analyze sperm function in our rat model. Data regarding the sperm function in patients with T1DM have been published multiple times for animal models and humans. Therefore sperm analysis would not have given us new insights or data to identify novel pathophysiological mechanisms. Germ cell markers were analyzed at the transcriptional level but showed no significant differences in expression. In the NT group, we found more abnormal tubuli and fewer tubuli with elongated spermatids. For both groups that were treated with insulin, we still found fewer tubuli with elongated spermatids and fewer tubuli in stages VII to XII. We do not believe that these effects are strong enough to reduce reproductive outcome fertility rates.

Data on Sertoli cell function are sparse in rats and absent in humans with T1DM. Trindade et al found vacuolization in Sertoli cells (59) and Ballester et al found affected expression levels of c-kit, insulin-like growth factor 1, insulin, and FSH receptors (10). At the transcriptional level we did not find significant differences for Sertoli cell–specific markers, and inhibin levels were not different between the groups. Therefore we suggest that Leydig cells are the targets and are negatively affected after diabetes manifestation.

Other investigators have also studied the molecular mechanisms behind the effects of T1DM on testis function in animal models. Some groups suggest that oxidative stress may play an important role in the pathophysiology of T1DM-related reproductive dysfunction and abnormalities (68-70). Nuclear factor-E2–related factor 2 (*NRf2*) expression, a gene for oxidative defense, was downregulated in diabetic rats (71, 72). In humans, sperm have a greater DNA-fragmentation index and increased advanced glycation end products and receptors (RAGE) that results in deterioration of sperm quality and changes in testicular metabolite levels and spermatogenic gene expression (24, 68-70). Antioxidant treatment may improve the glycemic index and can therefore reduce diabetic complications and protect against free radical–induced oxidative stress (73, 74). We found a strong and significant increase in ROS/RNS activity in the serum of NT rats. After insulin treatment this effect was completely reversible and there was no sign of oxidative stress in our rats. Even after a longer treatment duration, serum levels were unchanged. Our data therefore indicate there are signs of oxidative stress in the serum of diabetic rats, but it is unlikely one of the major factors underlying the observed findings in testicular steroidogenesis and spermatogenesis.

Unexpectedly after diabetes manifestation, we found significantly fewer apoptotic cells in the testis compared to HCs in the NT group and in the SIT group. We suggest that unknown protective mechanisms might be activated to save and protect negatively affected cells because of the lack of insulin. Directly after manifestation of diabetes, we observed decreasing intratesticular *TNF-α* expression, suggesting attenuation of an inflammatory process. Therefore, we can speculate that inflammation and apoptosis are not the pathophysiological mechanisms negatively affecting Leydig cell function in the acute phase after diabetes manifestation. We rather believe that the lack of insulin and the hyperglycemic state lead to an imbalance of metabolites that influences testicular metabolism and the blood-testis barrier.

After a longer treatment duration with insulin, we found the expected increase in apoptotic cells, which is in line with findings by others (42, 75). We speculate that the number of Leydig cells decreases because of the process of apoptosis. Long-term diabetes with chronically higher blood glucose levels might induce chronic inflammation. For example, Liu et al showed that T2DM mice presented with increased apoptosis and decreased autophagy in the testes (76). Those effects were nullified by metformin therapy. They speculated that metformin rescues diabetes-induced testicular damage by attenuating apoptosis and inducing autophagy. This effect is likely mediated by the PK2/PKR/AKT/GSK3β signaling pathway (76). The pathophysiological mechanism(s) might be similar in long-term T1DM and insulin treatment.

In line with published data, the *TNF-α* expression in the testes significantly increased in the long-term–treated rats. Taking inflammation as a possible explanation for long-term negative effects of diabetes, we speculate that this might be a possible reason for the significant upregulation of steroidogenic enzymes after a short treatment duration. This is in line with our study of rats with obesity. After the inflammation process has started, we also found an upregulation of the steroidogenic enzymes. We speculated that this might be a compensatory mechanism of the body trying to keep testosterone levels in the normal range (63). At some point this mechanism might fail and steroidogenic enzyme expression might possibly be reduced and serum and intratesticular testosterone levels might decrease. Therefore, it will be of great interest to analyze diabetic rats that have been treated for an even longer duration.

Our study has 2 limitations. After diabetes manifestation, rats had to be killed on average after 7 to 14 days because of severe symptoms and not at a defined time point. The group that was on treatment for 6 weeks was probably not treated long enough for us to evaluate long-term effects.

With our animal study, we had the chance to evaluate effects of T1DM at the testicular level and were able to show

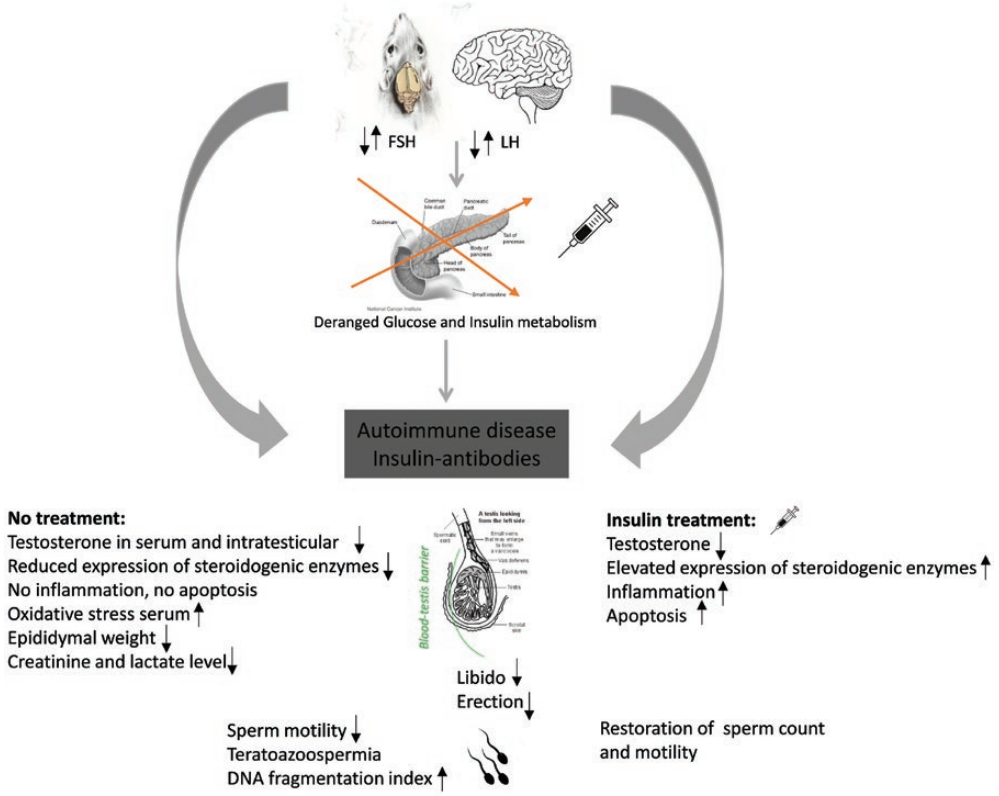


Figure 6. Schematic representation of the effects of type 1 diabetes mellitus (T1DM) on reproductive function in male rats. Effects of T1DM on the testicular function. This schematic representation demonstrated differences between no insulin treatment and insulin treatment. FSH, follicle-stimulating hormone; LH, luteinizing hormone.

that the damage is due to a combination of local effects from hyperglycemia and/or the lack of insulin and by alterations in hormone levels that disrupt the hypothalamic-pituitary gonadal axis (Fig. 6). The recent discovery that the testes and sperm both produce insulin brings a new perspective on how diabetes and insulin signaling at the testicular level may contribute to subfertility (9). We speculate that sexual dysfunctions, like reduced libido and erectile and ejaculatory dysfunction, which were just thought to be long-term complications of T1DM and induced by end-organ damage and neuropathy, might also partly be due to defects at the cellular, testicular, and endocrine-regulatory level.

Conclusion

In summary, diabetes manifestation with hyperglycemia and a severe lack of insulin leads to acute Leydig cell dysfunction and reduced differentiation. Steroidogenic enzymes are downregulated and serum and intratesticular testosterone levels are significantly reduced as a primary effect, together with inappropriate lowering of the gonadotrophins as an adjunct effect. This process was partially reversible with well-compensated blood glucose control. Long-term T1DM adversely affects Leydig cell function due to hyperglycemia, inflammation, and apoptosis.

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Additional Information

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Disclosures: The authors have nothing to disclose.

Data Availability: Most of the data generated or analyzed during this study are included in this published article or in the data repositories listed in "References." Only a few data sets generated during and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

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