

Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality

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BACKGROUND: The objective of the study was to investigate the hypothalamo-pituitary–testicular axis and sperm structure at the transmission electron microscope (TEM) level in men affected by insulin-dependent diabetes. **METHODS:** Twenty-two diabetic men and 24 controls were recruited. GnRH (100 µg) was administered and FSH- and LH-induced secretion was evaluated. Semen samples were collected and sperm concentration and motility were determined using a Makler chamber. Ejaculated sperm were fixed and observed with a TEM. **RESULTS:** The response of gonadotrophins to GnRH was significantly lower in diabetics than in control men. Sperm motility was also significantly lower. At the electron microscope level, sperm from diabetics exhibited a higher percentage of immaturity- and apoptosis-related defects than sperm from controls. **CONCLUSIONS:** The reduced response of gonadotrophins to GnRH in diabetic men may indicate a decreased acute releasable pool of pituitary gonadotrophins. The results of TEM examination showed that sperm from men with diabetes presented severe structural defects in comparison with sperm from controls. It is possible that the reproductive impairment recognized in men with diabetes could be the result of interference by the disease on the hypothalamo-pituitary–testicular axis at multiple levels, as indicated by the reduced gonadotrophin response to appropriate stimuli and by the abnormal ultrastructure of ejaculated sperm. The defective spermatogenesis may be the consequence of a direct testicular effect of the disease.

Key words: diabetes/GnRH/hypothalamus–pituitary–testicular axis/infertility/sperm structure

Introduction

Diabetes has been associated with reproductive impairment in both men and women. Although the pathophysiology of reproductive derangements in young diabetic women has been largely investigated, few studies have been conducted in men. In the rat model for diabetes, a reduced pituitary response to GnRH has been shown (Seethalaksmi *et al.*, 1987). Furthermore, elevated pituitary gonadotrophins have been found in diabetic rats in conjunction with an impaired pituitary response to exogenous GnRH (Bestetti *et al.*, 1985). In humans, the evidence indicates that diabetics display a blunted response of LH (Distiller *et al.*, 1975; Wright *et al.*, 1976; Djursing *et al.*, 1983, 1984) and FSH (Distiller *et al.*, 1975) to GnRH administration, although some studies failed to show such a reduced response (Rastogi *et al.*, 1974; Daubresse *et al.*, 1978). Some investigators have proposed that the neuroendocrine

lesion in diabetes is at the level of the hypothalamus (Bestetti and Rossi, 1982; Press *et al.*, 1984). This hypothesis has been strengthened by the recent observation that insulin infusion in the lateral ventricle of streptozotocin-induced diabetic sheep increases the LH pulse frequency (Tanaka *et al.*, 2000). Alterations in carbohydrate homeostasis, such as diabetes, have been associated with disturbances of the functional activity of the reproductive system in laboratory animals, not only of the hypothalamo-pituitary axis, but also of gonads.

It is known that well-controlled diabetes is associated with a physiological endocrine testicular function, as shown by plasma androgen levels in the physiological range (Handselman *et al.*, 1985). However, a large body of evidence has demonstrated that diabetic men appear to be at a disadvantage in terms of sperm quality compared with healthy controls (Handselman *et al.*, 1985; Vignon *et al.*, 1991; Ali *et al.*, 1993a; Niven *et al.*, 1995)

The objective of the present study was to investigate the hypothalamo-pituitary–testicular axis and sperm quality in men affected by insulin-dependent diabetes.

Materials and methods

Twenty-two diabetic infertile men were recruited from patients attending the Centre for the Study of Germinal Cells, Siena, between 1998 and 2001. The mean age of patients (\pm SD) was 38 ± 6 years (range 30–45) and the mean duration of the disease was 11.3 ± 8.0 years. All patients were treated with insulin, were normotensive, had good kidney function and did not suffer from ketoacidosis. Men with hypergonadotrophic hypogonadism were excluded. No patients had signs of neuropathy or were impotent. Impotence was defined as difficulty in obtaining erection suitable for vaginal penetration for a minimum period of 6 months. Diabetic men free of renal disease, haemochromatosis, or any medication other than insulin were selected. The metabolic control of the diabetes was assessed by HbA1c analysis. The mean HbA1c value in diabetic patients was $9.1 \pm 3.0\%$ (range 5.0–13.4).

Twenty-four healthy men (controls) were selected from a large group of non-diabetic infertile men in our database. They were healthy with a mean age of 37 ± 5 years (range 31–46).

Before performing the study, complete history, physical examination and relevant laboratory makeup were performed. Exclusion criteria were history of drug or alcohol abuse, ongoing medical treatment with anabolic steroids and gonadotrophins, heavy smoking habit (>10 cigarettes/day), hypertension, leukocytospermia, varicocele and unilateral testicular atrophy.

Patients and controls underwent a GnRH test and semen examination with optic and electronic microscopy. Written informed consent was obtained from all men. Patients and controls were admitted to the Gynecological Endocrinology Hospital Center of Siena University 2 h before blood sampling was to begin. An indwelling catheter was inserted in the antecubital vein and saline solution was infused slowly to keep the vein patent. All patients did the releasing hormone test (100 μ g GnRH; Biochem. Immunosystems, Milan, Italy).

After 2–3 days of sexual abstinence, semen samples were produced by masturbation, collected into sterile specimen cups and allowed to liquefy at room temperature. Semen volume, sperm concentration and motility were determined according to World Health Organization guidelines (World Health Organization, 1999). Sperm concentration and motility were determined by analysing 5 μ l of semen using a Makler chamber (Makler, 1980).

Transmission electron microscopy (TEM) procedure

Ejaculated sperm were fixed in cold Karnovsky's fixative and maintained at 4°C for 2 h. The fixed semen was then centrifuged at 1500 *g* for 15 min. The pellet was removed from the centrifuge tubes, washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, post-fixed for 1 h at 4°C in 1% buffered osmium tetroxide, dehydrated and embedded in Epon Araldite. The sections, cut with a Supernova ultramicrotome (Reichert Jung Wien, Austria), were collected in copper grids, stained with uranyl acetate and lead citrate, observed and photographed with Philips CM 10 electron microscope (Philips Scientific Instruments, Eindhoven, The Netherlands). For each subject, 300 sperm were randomly observed. Two highly trained evaluators, who were blinded to the aim of the study, performed the TEM analysis. A total of 300 sperm sections were randomly evaluated in at least three different embedded samples for each ejaculate.

TEM data were statistically evaluated by the computerized formula of Baccetti *et al.* (Baccetti *et al.*, 1995). Briefly the formula evaluates structural and functional sperm integrity by computerizing the submic-

Table I. Demographic, hormonal and semen characteristics of diabetics and healthy controls

Variables	Diabetics (<i>n</i> = 22)	Controls (<i>n</i> = 24)	<i>P</i> -value
Age	38 \pm 6	37 \pm 5	NS
BMI (kg/m ²)	26 \pm 4	27 \pm 3	NS
Smoking (yes/no, %)	23	25	NS
LH (mIU/ml)	3.5 \pm 0.8	3.1 \pm 0.8	NS
FSH (mIU/ml)	3.7 \pm 0.5	3.5 \pm 0.7	NS
Testosterone (pg/ml)	6800 \pm 2000	7200 \pm 2100	NS
Free testosterone (pg/ml)	24 \pm 7	28 \pm 6	NS
Seminal fluid (ml)	3.3 \pm 1.2	2.1 \pm 1.2	NS
Concentration (10 ⁶ /ml)	43.4 \pm 30	65 \pm 50	NS
Total sperm number (10 ⁶)	136 \pm 89	201 \pm 190	NS
Motile sperm (a+b, %)	18 \pm 11	60 \pm 12	0.0001

Data are mean \pm SD.

NS = not significant; BMI = body mass index.

To convert values for LH and FSH to units per litre, multiply by 1; to convert values for free and total testosterone to pmol/l multiply by 3.47.

roscopical characteristics detected by TEM. The formula is valid for a real system of 15 variables and allows a precise simultaneous evaluation of the sperm characteristics and a determination of the total number and percentage of sperm free from ultrastructural defects (Baccetti *et al.*, 1995).

Hormone assay

Plasma FSH, LH, testosterone and free testosterone levels were assayed by double-antibody radioimmunoassay using commercial kits from Radim (Rome, Italy) for FSH and LH, from Sorin (Saluggia-VC, Italy) for testosterone, and from DPC (Los Angeles, CA, USA) for free testosterone. Samples were assayed in duplicate at two dilutions. Samples from a given subject were analysed for each hormone in the same assay to avoid inter-assay variation. Quality control pools at low, normal and high LH, FSH, testosterone and free testosterone concentrations were present in each assay. The detection limit of the assay was 0.20 IU/l for LH, 0.18 IU/l for FSH, 277 pmol/l for testosterone and 0.5 pmol/l for free testosterone. Intra- and inter-assay variations were 7.8 and 8.2% for LH, 6.2 and 6.5% for FSH, 3.4 and 4.6% for testosterone, and 4.6 and 4.7% for free testosterone.

Statistical analysis

Results are expressed as means \pm SD. The total integrated hormonal responses to GnRH were calculated by the trapezoidal method and expressed as the area under the concentration–time curve (AUC). To compare the differences between the two groups, peak values (the maximum rise above baseline value) and AUC were compared. Comparisons between the two groups were calculated by unpaired *t*-test if the data were normally distributed and by the Mann–Whitney *U*-test because of the small size of the groups. Statistical analysis was performed with the Statsoft software. Statistical significance was set at $P < 0.05$.

Results

Demographic, hormonal and seminal characteristics of patients and controls are reported in Table I. There were no significant differences in basal hormonal levels between the two groups. No differences were observed in seminal fluid volume, sperm concentration or total sperm number. Sperm motility was significantly lower in diabetic than in healthy men (18 ± 11 versus $60 \pm 12\%$; $P < 0.05$).

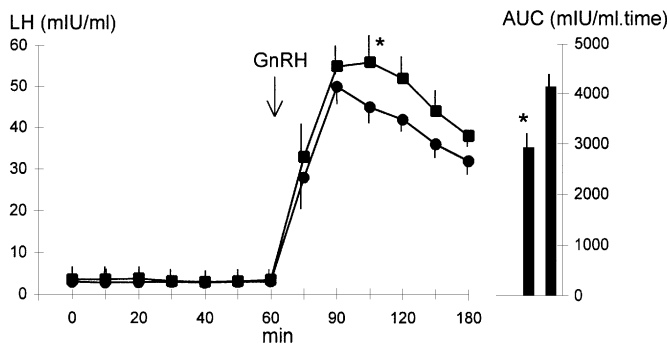


Figure 1. LH response to GnRH administration. Peak response of LH and AUC were lower in men with diabetes (●) than in controls (■) (**P* < 0.05).

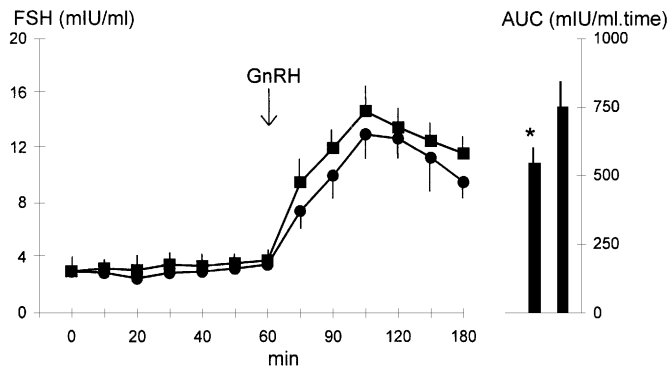


Figure 2. FSH response to GnRH administration. AUC were lower in men with diabetes (●) than in controls (■) (**P* < 0.05).

Results of GnRH administration were available for 13 patients and 14 controls. There were no significant differences in serum levels of LH, FSH, testosterone or free testosterone between diabetics and controls. GnRH administration was followed by a lower LH response in diabetics than in healthy men (48 ± 8 versus 59 ± 10 mIU/ml; *P* < 0.05) (Figure 1). The peak response of FSH to GnRH administration was not significantly lower in diabetics than in controls; however, AUC FSH was significantly lower in men with diabetes than in healthy men (540 ± 68 versus 740 ± 75 mIU/ml.time; *P* < 0.05) (Figure 2).

Sperm structure by TEM was available for nine patients and 10 controls. Ultrastructure was evaluated by the formula of Baccetti *et al.* (Baccetti *et al.*, 1995) and it was found that the number of sperm devoid of ultrastructure defects was lower in diabetics than in controls (0.529 ± 0.386 versus $26.67 \pm 19.5 \times 10^6$; *P* < 0.05). Immaturity- and apoptosis-related defects were evident (Figure 3). Acrosomes showed abnormal shape in 78% and reduced dimension in 57% of sperm. Nuclear shape was abnormal in 74% of cases. Incorrectly assembled mitochondria were present in 45% of sperm. The plasma membrane was broken in 32% of germinal cells. The comparison of sperm structure of diabetics and controls is reported in Table II.

Discussion

Conflicting data have been published on hypothalamo-pituitary–testicular function in diabetic patients. In comparison with healthy subjects, a blunted response of LH (Distiller

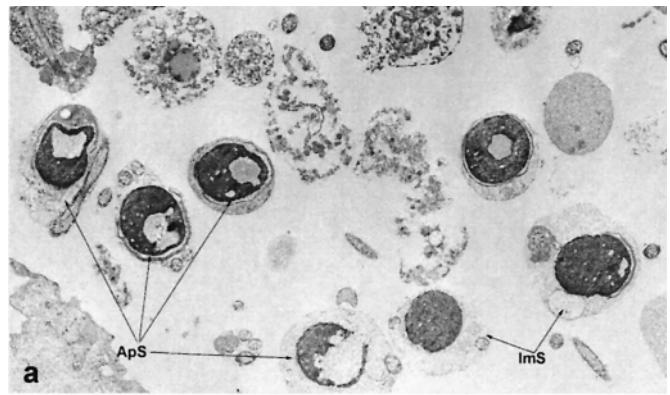


Figure 3. TEM micrograph of longitudinal and cross-sections of sperm from diabetics patients. Apoptotic sperm and spermatid (ApS) are characterized by irregularly shaped nuclei with margined chromatin. Immature germ cells (ImS) show elliptical or round nuclei with uncondensed chromatin and cytoplasmic residues embedding coiled axonemes. Magnifications: (a) $\times 3200$; (b) $\times 6300$.

et al., 1975; Wright *et al.*, 1976; Djursing *et al.*, 1983, 1984; Vierhapper, 1985) and FSH (Distiller *et al.*, 1975) has been described by some, but not all, investigators (Rastogi *et al.*, 1974; Daubresse *et al.*, 1978; South *et al.*, 1993). These conflicting data may result from the inhomogeneity within the small groups of patients included in the study, the duration of diabetes, or the extent of concomitant gonadal impairment.

Insulin has been shown to play a central role in the regulation of pituitary and gonadal function. Indeed, insulin enhances LHRH-induced gonadotrophin secretion *in vitro* (Adashi *et al.*, 1981). A reduced glucose utilization by the anterior pituitary cells (Goodner and Freinkel, 1961; Garris *et al.*, 1984) and a decreased response of FSH and LH to GnRH administration (Kirchick *et al.*, 1979; Bestetti *et al.*, 1985; Seethalakshmi *et al.*, 1987) has been shown in insulin-deficient rats.

Table II. Sperm ultrastructure in diabetics and healthy controls

Sperm ultrastructure characteristics	(%)	Diabetics (n = 9)	Controls (n = 10)	P-value
Status of sperm	Single	95 ± 5	96 ± 4	NS
Acrosome	Normal position	56 ± 23	87 ± 7	0.001
	Normal shape	22 ± 12	46 ± 10	0.0001
	Normal dimension	43 ± 14	67 ± 13	0.0023
	Compact content	56 ± 18	84 ± 6	0.0001
Nucleus	Normal shape	26 ± 15	55 ± 6	0.01
	Compact	28 ± 12	55 ± 7	NS
Chromatin	Marginated	19 ± 9	4 ± 2	NS
	Uncondensed	47 ± 12	37 ± 11	NS
	Absent	72 ± 12	86 ± 15	NS
Cytoplasmic residues	Absent	72 ± 12	86 ± 15	NS
Mitochondria	Regular shape	65 ± 20	71 ± 18	NS
	Regular helix assembly	55 ± 22	70 ± 18	NS
Axoneme	Normal pattern	54 ± 9	70 ± 17	NS
	Normal arms	44 ± 26	53 ± 32	NS
	Normal shape	64 ± 13	83 ± 15	0.014
Accessory fibers	Normal structure	40 ± 16	64 ± 15	0.012
Plasma membrane	Normal structure	68 ± 20	76 ± 11	NS
'Healthy sperm' (10 ⁶)		0.529 ± 0.386	26.67 ± 19.5	0.0001
Apoptosis probability		14 ± 7	5.1 ± 2.5	0.0011
Immaturity probability		72 ± 10	51 ± 22	0.011
Necrosis probability		40 ± 18	34 ± 12	NS

Data are mean ± SD.

NS = not significant.

Animal experiments have indicated a reduced gonadotrophin secretion in chemically induced diabetes either due to inadequate GnRH release (Johnson and Sidman, 1979; Rossi and Bestetti, 1981) or reduced pituitary responsiveness to GnRH (Kirchick *et al.*, 1979; Dong *et al.*, 1991). Diabetic rats showed an abnormal sexual steroid feedback on the hypothalamo-pituitary axis either due to reduced pituitary sensitivity (Kirchick *et al.*, 1979; Dong *et al.*, 1991) or abnormal steroid transport into the effector cells (Gentry *et al.*, 1977)

The reduced LH and FSH response to GnRH in diabetic men indicated a decreased acute releasable pool of pituitary gonadotrophins. In diabetic women, a reduced LH response to GnRH and alterations in LH secretory activity have been recognized (Djursing *et al.*, 1983, 1984; la Marca *et al.*, 1999). Furthermore, increased opioidergic and dopaminergic tones have been hypothesized. Increased central opioid and dopamine have a deleterious effect on reproductive function by acting mainly on the GnRH-LH axis.

We have demonstrated that diabetes in women is associated with a subclinical hypercortisolism, which in turn should have a role on reproductive impairment (la Marca *et al.*, 1999). Whether these mechanisms are functional in diabetic men is not known.

As for hormonal evaluation, discordant data have been published on sperm quality in diabetic men. In the present study, we have found reduced sperm motility in diabetic men by examination with a Makler chamber. For diabetics, a higher sperm count, higher sperm concentration (Padron *et al.*, 1984; Vignon *et al.*, 1991; Ali *et al.*, 1993), higher percentage of abnormal sperm (Padron *et al.*, 1984; Vignon *et al.*, 1991) and lower motility (Padron *et al.*, 1984; Handelsman *et al.*, 1985; Ali *et al.*, 1993; Niven *et al.*, 1995) have been reported. Others found reduced sperm volume and normal sperm density, morphology and motility (Handelsman *et al.*, 1985).

The reasons for the discrepancies between the studies can probably be found, as for the hormonal evaluations, in the limited study population. To our knowledge, this is the first paper to investigate sperm structure in diabetic patients. Although limited to few patients, results of TEM examination showed that sperm from men with diabetes presented severe structural defects. In particular, apoptosis- and immaturity-related defects were recognized. Indeed, alterations in the acrosome, nucleus, mitochondria and plasma membrane were observed. Using the mathematical formula by Baccetti *et al.* (Baccetti *et al.*, 1995), we found that the percentage of sperm devoid of ultrastructure defects in diabetics was very low (mean 0.4%).

The results of the TEM examination indicate a role for insulin and carbohydrate metabolism in spermatogenesis. The evidence indicates that, at least in animals, insulin plays a role in the maintenance of spermatogenesis and testicular endocrine function. Studies in streptozotocin-induced diabetic rats showed severe dysfunction of the reproductive tract. Diabetic rats had decreased reproductive organ weights as well as diminished sperm counts and motility (Seethalakshmi *et al.*, 1987).

Male diabetic rats showed extensive spermatogenic alterations when electron microscopy was used (Gondos and Bevier, 1995). The same animals, when insulin-treated, showed variable changes ranging from normal spermatogenesis to moderate or severe alterations, indicating that in diabetic rats insulin could, even if not entirely, prevent spermatogenic abnormalities (Gondos and Bevier, 1995)

Reproductive function damage associated with diabetes is far from understood. A chronic subtle impairment of gonadotrophin secretion may, at least in part, be responsible for defective spermatogenesis. However, the reduction in LH and FSH responses to GnRH administration is too small to be

considered as a primary event in causing disturbances in sperm structure. In our opinion, defective spermatogenesis may be the consequence of a direct testicular effect of the disease.

In conclusion, it is possible that the reproductive impairment recognized in men with diabetes could be the result of interferences of the disease on the hypothalamo-pituitary-testicular axis at multiple levels, as indicated by the reduced gonadotrophin response to appropriate stimuli and by the abnormal ultrastructure of ejaculated sperm.

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