Antioxidants Attenuate the Effects of Insulin Dependent Diabetes Mellitus on Sperm Quality

Omu AE, Al-Bader MD, Al-Jassar WF, Al-Azemi MK, Omu FE, Mathew TC and Anim JT

1Department of Obstetrics and Gynaecology, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait
2Department of Physiology, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait
3Department of PAAET, College of Nursing, Kuwait University, Kuwait
4Department of Anatomy, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait
5Department of Pathology, Faculty of Medicine, Health Sciences Center, Kuwait University, Kuwait

Abstract

Introduction: The prevalence of Diabetes Mellitus has been increasing in an epidemic proportion worldwide and it is associated with impairment of sperm quality and cause infertility. The role of antioxidants therapy to improve human sperm quality has not been established.

Objective of study: To investigate the effect of antioxidants therapy on sperm quality in men with insulin dependent diabetes mellitus.

Materials and methods: Forty-five men with insulin dependent diabetes attending the andrology clinic, between January 2008 and December 2012 seen at the Maternity Hospital, Kuwait, form the subjects of this study. Thirty non-diabetic infertile men matched for age and duration of infertility formed the control group. The study protocol included initial pretherapy and post-therapy clinical evaluation of all the patients, semen analysis, hormone profile, glycosylated haemoglobin (HbA1C), Malonedialdehyde (MDA), lipid profile, Acridine orange denaturation of sperm for evaluation of sperm DNA fragmentation index and light and electron microscopy. The patients were administered Zinc, Selenium and vitamins E and C for three months and revaluated.

Results: Diabetes mellitus was associated with significantly impaired sperm motility (asthenozoospermia) compare to control (64% versus 36%) (P<0.05), normal sperm morphology (66% versus 52%) (p<0.05), higher HbA1C (9.6% versus 4.4%, P<0.05) and oxidative stress (MDA) (2.4 versus 1.4 nmol/L, P<0.01) and reduced antioxidant status. Antioxidant therapy significantly decreased glucose level, 18-40% p<0.05; HbA1C 9-20% p<0.05; MDA level 33-41%, P<0.01; and Sperm DNA Fragmentation index, 23-33%, p<0.01) and Increase in BuChE 21-40%, p<0.05 and TAC, 27-36%, p<0.05.

Conclusion: Diabetes mellitus particularly with poor glycomic control is associated with impaired sperm quality, involving oxidative stress in the pathogenesis. Antioxidant therapy has been shown to significantly improve the sperm quality.

Keywords: Diabetes mellitus; Oxidative stress; Sperm quality; Antioxidant

Lay summary

This study was conducted to evaluate the protective effects of antioxidants on sperm quality of men with insulin dependent diabetes mellitus. The study involved 45 diabetic men on insulin therapy and 30 non-diabetic infertile men as controls. Both groups were seen and evaluated the infertility clinic of the Maternity Hospital, Kuwait. They had semen analysis and estimation of lipid and hormone profiles, malonedialdehyde (MDA) a marker of oxidative stress, glycosylated haemoglobin (HbA1C) (with low value of below 7% as evidence of good control), and sperm DNA damage. Both groups of men were administered Zinc, selenium, vitamins E and C for three months. The investigations above were then repeated.

The results of this study showed that diabetes mellitus was significantly associated with impaired sperm motility and abnormal sperm morphology, higher glycosylated haemoglobin, oxidative stress and sperm DNA damage. Interestingly, after three months of administration of antioxidants there was decreased serum glucose level, oxidative stress (MDA), HbA1C, sperm DNA damage and increased total antioxidant activity and obvious improvement of sperm parameters. We advocate dietary consumption of food rich in antioxidants, especially fruits and leafy vegetables that are rich in natural antioxidants and antioxidant supplementation by diabetic men, to prevent the oxidative effect of diabetes on sperm to cause infertility and other non-fertility complications in diabetic men.

Introduction

Diabetic epidemic will continue to rise and in 2030 will be 4.4% from 2.8% in 2000 and the number of diabetes will reach 366 million by 2030 [1], with potentially devastating effect and expensive treatment. More recent global estimates involving 216 countries put prevalence of diabetes of diabetes in the adult population at 6.4% affecting 285 million in 2010 to 7.7% in 439 million adults by 2030 [2]. Current
The increasing incidence of DM worldwide will inevitably result in a higher prevalence of diabetes related infertility [20]. A recent study reported that 35 percent of type 2 diabetes is infertile [12]. Young male diabetic patients are likely to present high infertility/subfertility prevalence resulting from impaired reproduction function and poor semen quality [21].

**Objective of study**

To investigate the effect of antioxidants on improving the adverse effect of poor glycemic control on sperm quality in males with insulin dependent diabetes mellitus with operative hypothesis that zinc, selenium and antioxidants vitamins C and E may have protective effects on oxidative stress induced impairment of the sperm parameters of diabetic men.

**Materials and Methods**

Forty five men with insulin dependent diabetes and thirty non-diabetic infertile men as control, attending the combined infertility clinic, between January 2008 and December 2012 at the Maternity Hospital, Kuwait were evaluated. Ethical approval by the institutional board of the Faculty of Medicine Kuwait University was obtained before the commencement of the study.

To be included in the study, the men must have been cohabiting with their spouses without the use of contraception for at least 12 months, nonsmokers and not on drugs for/and history of chronic diseases for the past 6 months, blood pressure less than 140/90 mm Hg and body mass index ≤ 30 kg/m². All the diabetic patients were on insulin at consultation or were commenced on insulin after referral to the diabetic clinic.

Thirty men in control group were matched for age, duration of infertility and body mass index with the study, cohabited with their spouses for at least 12 months or more and not on drugs for chronic condition like hypertension. All the men had normal fasting blood glucose of <5.3 mmol/l to make sure they were not diabetic.

The metabolic control of the diabetes was assessed by HbA₁c. The study protocol included initial clinical evaluation of both patients and controls. Semen analysis, lipid and hormone profiles: LH, FSH and Testosterone; HbA₁c; total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX); Malondialdehyde (MDA), electron microscopy of sperm and Sperm DNA fragmentation were carried out before and after the patients were treated with Zinc 250 mg, Selenium 300 μg and vitamins E 20 mg and C 10 mg twice daily for three months.

**Semen analysis**

After 3-day-sexual abstinence, semen samples produced by masturbation were collected into sterile specimen cups and allowed to liquefy at room temperature. Semen analysis was determined according to WHO guidelines [22] using 5 µl of semen on a Makler chamber.

**Blood sample preparation**

Blood was collected from patients after overnight fast by venipuncture into EDTA tubes underarm separated by density centrifugation using a Ficoll-Paque/EPlus centrifuge (Pharmacia Biotech, Uppsala, Sweden) and stored at -20°C for testosterone, Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) levels measured by radioimmunoassay and lipid profiles, and for malondialdehyde (MDA), and superoxide dismutase (SOD), glutathione...
peroxidase (GPX) and total antioxidant capacity (TAC).

**Estimation of MDA a marker of Oxidative Stress**

For MDA estimation, into 1.0 ml of serum, 0.5 ml of 350 g/L trichloroacetic acid (TCA), and 1.0 ml of 0.5% thiobarbituric acid were added and mixed. The mixture was incubated at 60°C for 90 min. After cooling at room temperature, 1.0 ml of 700 g/L TCA and 2.0 ml of chloroform were added, mixed and centrifuged at 1500 g for 20 min. The absorbancy of the sample supernatant was measured at 532 nm.

**Enzymatic estimation of antioxidants**

SOD, GPX and TAC were estimated colorimetrically using a commercially available kit (Randox Lab, UK) strictly according to the manufacturer’s instructions.

Glucose was measured by the hexokinase method (Glucocount; Boehringer-Mannheim).

**Estimation of lipid profile**

Five ml of venous blood samples were drawn from all subjects under all aseptic precautions. Thereafter, the blood was allowed to clot (for 10 min) and serum was separated by centrifugation at 2500 rpm for 20 min. Each serum sample from different groups was evaluated for using diagnostic kit for Total cholesterol (mg/dl), Triglyceride (mg/dl) and HDL-cholesterol (mg/dl). LDL-cholesterol (mg/dl) and VLDL-cholesterol (mg/dl) were calculated using Friedewald formula.

**Chromatographic method for HbA₁c**

The chromatographic assay uses an HPLC instrument (LC module) with pump, injector and UV detector of 292 nm filter (Millipore). Enzymatic estimation of Antioxidants

Chromatographic method for HbA₁c

The serum levels of FSH, LH and Testosterone were determined in the blood by an in vitro assay.

**Sperm DNA fragmentation index**

This was determined by Sperm chromatin Structure Assay (SCSA) using Acidine orange denaturation as described by Evenson and Jost [23] and assessed with fluorescence microscopy. Two to three hundred spermatzoa denatured by acidine orange (AO) were counted and percentage of red colored spermatzoa designated as sperm DNA fragmentation (DFI).

**Histology:** Formaldehyde-fixed semen samples were embedded in paraffin and then sliced (slice thickness, 3–4 µm) on silane-precoated slides, deparaffined with xylol, and histologic observations were performed after staining by the hematoxylin-eosin method and assessed with light microscopy.

**Electron Microscopy of Spermatozoa:** Semen samples were washed three times with phosphate buffer (0.1 mol/L, pH 7.4), pelleted by centrifugation, fixed in 3% glutaraldehyde, followed with 1.3% osmium tetroxide, then embedded in Epon Araldite and section photographed by a Seiss 109 Electron Microscope (Zeiss Oberkohen, Germany) after double staining with uranyl acetate and lead citrate.

**Statistical analysis:** Data entry was carried out on SPSS version 17, with release 4.1/4.0 for logistic regression and one way analysis
of variance. Results are expressed as means ± SEM. Levene analysis of variance and Student’s t and z tests for paired data were used to determine the significance of differences between pre and post-therapy results. The level of statistical significance was set at P<0.05 for multiple comparisons. All analyses were performed on StatView 5.0 for the Macintosh (Abacus Concepts, Berkeley, CA).

Results

The patients were on two main types of insulin, namely Intermediate acting (NPH) insulin take twice daily and long acting taken once daily. There were no significant differences in the age and duration of diabetes mellitus between the two study groups and controls. The mean HbA1c value in diabetic patients was 8.8 ± 4.1% (range 4.0-13.4) versus 4.6 ± 2.8%, for the control group (P<0.05). With the diabetic men structured according to their level of HbA1c (<7% for good glycemic control and ≥ 7% for poor glycemic control, 42.2% (19/45) had poor glycemic control. The prevalence of poor glycemic control was higher at 51.1% if the new IDA cut-off of 6.5% or higher for glycemic control.

Effect of diabetes mellitus on sperm parameters

As shown in Table 1, men with diabetes were more significantly associated with poor sperm parameters compared to the control patients (P<0.05). Similarly, poor diabetic control in form of HbA1c ≥ 7% was significantly associated with impaired sperm motility; progressive motility, p<0.05, asthenozoospermia (P<0.01) and normal morphology (p<0.02). Similarly, men with ≥ 7% (HbA1c), were significantly associated with abnormal lipid profile; cholesterol (p<0.05), triglyceride (p<0.05), LDL (p<0.01) and lower HDL (p<0.05). There was no association between glycemic control and FSH and LH. However Testosterone was higher in the control group than in the study diabetic group (P< 0.01) and reduced with poor diabetic control (P<0.05).

Figure 1 show typical micrograph of Haematoxylin and Eosin staining of control and diabetic patients. Figure 2 staining pattern of Acridine orange acid denaturation of sperm DNA, to evaluate sperm DNA fragmentation, and Figure 3 for evaluation of sperm morphology by transmission electron microscopy. As shown in Table 2 and Figure 4, there was a strong association between poor diabetic control (HbA1c ≥ 8%) and sperm defects (teratozoospermia); double head (p<0.05), round and elongated spermatozoon (p<0.05) and cytoplasmic mid tail piece (p<0.05). Leukocytzoospermia, an index of inflammatory process was also more common in diabetic men with HbA1c ≥ 7%.

Antioxidants therapy was associated with improved sperm quality as shown in Table 3 in form of sperm count, 47-57%, (P<0.01); progressive motility, 35-38%, (P<0.01); asthenozoospermia, 44-48%, (p<0.01) and normal sperm morphology,16-26%, (P<0.05). The differences observed were much stronger when diabetic men with ≥ 7% HbA1c were compared with those with initial glycemic control (<7% HbA1c) as shown in Figure 5.

In Figure 6, effect of antioxidant therapy is compared between poor glycaemic control versus good control. As shown in Table 3, there was positive correlation between serum glucose level and HbA1c (r=0.625, p<0.001), MDA (r=0.524, p=0.01) and Sperm DNA fragmentation index (r=0.482, p<0.05) and an inverse relationship with total antioxidant capacity (TAC) (r=-0.482, p<0.05). Antioxidant therapy significantly decreased glucose level, 18-40% (P<0.05); HbA1c 9-29% (P<0.05); MDA level 33-41%, (P<0.01); and Sperm DNA Fragmentation index, 23-33%, (p<0.01) and increase in TAC, 27-36%, (p<0.05).

Discussion

Effect of insulin dependent diabetes on human spermatozoa

The significant findings in the present study include the association between men with insulin dependent diabetes and impairment of quality of sperm such as asthenozoospermia and teratozoospermia that
Figure 2a: Acridine Orange denaturation in Control  
Figure 2b: Acridine sperm denaturation in DM

Acridine denaturation of ejaculated human sperm. The range is from Green (normal sperm) (NS) as shown in
(a) Control group with mainly green stained sperm (b) A typical diabetic patient with denatured sperm stained red (RS) (Sperm DNA fragmentation) with yellow-red, showing mild to moderate denaturation (Sperm DNA fragmentation in DM).

| Sperm Abnormalities | All Diabetics N=45 | Diabetic A N=26 | Diabetic B N=19 | Controls N=30 | P Value |
|---------------------|-------------------|----------------|-----------------|---------------|---------|
| Double head         | 11 (35.5)         | 5 (27.8)       | 6 (46.2)        | 4 (16)        | 0.02    |
| Large head          | 5 (29.0)          | 4 (22.2)       | 5 (38.5)        | 5 (20.0)      | 0.05    |
| Round spermatid     | 8 (25.8)          | 4 (22.2)       | 4 (30.8)        | 2 (8.0)       | 0.01    |
| Elongated spermatid | 8 (19.4)          | 3 (16.7)       | 5 (23.0)        | 1 (4.0)       | 0.04    |
| Cytoplasmic mid piece | 8 (25.8)       | 3 (16.7)       | 5 (38.8)        | 1 (4.0)       | 0.01    |
| Cytoplasmic tail    | 4 (12.9)          | 2 (11.1)       | 2 (15.4)        | 1 (4.0)       | 0.05    |
| Leukocytospermia    | 10 (32.3)         | 4 (22.2)       | 6 (46.2)        | 2 (8.0)       | 0.01    |

Table 2: Comparison of sperm abnormalities in semen in diabetic men and non diabetic controls
Diabetic A- Men with insulin dependent diabetes mellitus with HbA1c <7% versus Diabetic B- Men with insulin dependent diabetes mellitus with HbA1c ≥7%

| Sperm Abnormalities | All DM N=45 | A1 N=26 | B1 N=19 | All DM-2 N=41 | A2 N=23 | B2 N=18 | A2-A1 (%) | B2-B1 (%) |
|---------------------|-------------|---------|---------|---------------|---------|---------|-----------|-----------|
| Semen volume (ml)   | 3.6         | 3.6     | 3.6     | 3.7           | 3.8     | 3.6     | 2.8       | 5.6       |
| Sperm count (million/ml) | 18.4       | 19.2    | 18.6    | 28.6          | 30.2    | 27.4    | 7.3       | 47.3      |
| Asthenozoospermia (%) | 64.0        | 58.0    | 74.0    | 36            | 32.4    | 38.6    | 44.1      | 47.8      |
| Normal morphology (%) | 58.2        | 62.3    | 52.4    | 68.0          | 72.2    | 65.8    | 15.9      | 25.6      |

Table 3: Effect of antioxidants on sperm parameters in men with insulin dependent diabetes

Four men did not complete the final phase of the study and they were therefore not included in the post therapy analysis.

| Sperm parameter | All DM-1 | A1 | B1 | All DM-2 | A2 | B2 | A2-A1 (%) | B2-B1 (%) |
|-----------------|----------|----|----|----------|----|----|-----------|-----------|
| Semen volume (ml) | 3.6 | 3.6 | 3.6 | 3.7 | 3.8 | 3.6 | 2.8 | 5.6 |
| Sperm count (million/ml) | 18.4 | 19.2 | 18.6 | 28.6 | 30.2 | 27.4 | 7.3 | 47.3 |
| Asthenozoospermia (%) | 64.0 | 58.0 | 74.0 | 36 | 32.4 | 38.6 | 44.1 | 47.8 |
| Normal morphology (%) | 58.2 | 62.3 | 52.4 | 68.0 | 72.2 | 65.8 | 15.9 | 25.6 |
Diabetic A- Men with insulin dependent diabetes mellitus with HbA1c <7% versus Diabetic B- Men with insulin dependent diabetes mellitus with HbA1c ≥7%

There are significant changes in the diabetic men except with glutathione peroxidase

| Glucose (mmol/L) | Control N=30 | A1- HbA1c <7% | B1- HbA1c ≥7% | A2- HbA1c <7% | B2- HbA1c ≥7% | A2-A1 (%) | B2-B1 (%) |
|------------------|--------------|---------------|---------------|---------------|---------------|------------|------------|
|                  | 4.8±1.8      | 7.6±0.8       | 11.4±2.3      | 6.2±0.8       | 6.8±1.2       | 18.4       | 40.3       |
| TAC (mmol/L)     | 4.8±1.4      | 3.3±1.2       | 2.2±1.2       | 6.2±1.4       | 3.0±1.8       | 27.3       | 36.4       |
| SOD (mmol/L)     | 2.8±1.2      | 1.8±0.8       | 1.4±0.8       | 2.4±1.2       | 1.9±1.2       | 33.3       | 35.7       |
| GPX (mmol/L)     | 2.2±0.8      | 2.0±0.8       | 2.0±0.8       | 2.2±1.2       | 2.2±1.2       | 10.0       | 10.0       |
| HbA1c %          | 14.4±0.8     | 6.4±2.1       | 9.6±1.8       | 5.8±1.4       | 6.8±1.4       | 9.4        | 29.2       |
| MDA (mmol/L)     | 1.4±0.4      | 1.8±0.5       | 2.4±0.8       | 1.2±0.4       | 1.4±0.6       | 33.3       | 41.2       |
| DFI %            | 8.0±2        | 11.4±2.5      | 14.2±3.4      | 8.8±3.3       | 10.4±3        | 22.6       | 33.3       |

There are more significant reduction in oxidant and increase in antioxidant status with initial poor glycaemic control (HbA1c ≥7%).

Table 4: Association between glucose level, HbA1c antioxidant status and sperm DNA fragmentation index

**Figure 3a:** x 400 Transmission Electron Microscopy of a non-diabetic patient (control) with normal sperm head (NH) mid-piece with abundant mitochondria and normal tail (NT) and normal cross section with 9-2 n fibrils.

**Figure 3b:** Transmission Electron Microscopy of ejaculated semen of an insulin dependent diabetic man with infertility showing globular head (GH), cytoplasmic mid-piece with scanty mitochondria, elongated (SSp) and round Spermatid (RSp), Secondary spermatocyte and apoptotic body (Ap).

**Figure 3c:** Showing disruption of mitochondria and axonemal sheath and apoptotic bodies in a typical diabetic patient.

**Figure 4:** Sperm abnormalities. Sperm abnormalities in control non-diabetic compared with all diabetics, and poor glycaemic control. Double, round and globular sperm heads, elongated spermatid, cytoplasmic tail and leukocytospermia are more significantly common in the diabetic than control (P<0.01) and more common with poor diabetic (HbA1c ≥7%) than good control (HbA1c <7%) (P<0.05).
Figure 5: Effect of Antioxidant therapy on sperm parameters in men with insulin dependent diabetes mellitus. Comparison of the outcome of antioxidant therapy between poor glycaemic control (HbA1c ≥7%) and good glycemic control (HbA1c <7%). There is improvement all sperm parameters. Initial good glycemic control is associated with more significant improvement with sperm count (P<0.01) and progressive motility (P<0.05). Initial poor glycemic control shows more improvement with seminal volume (P<0.05), reduction of asthenozoospermia and normal sperm morphology (P<0.05).

Figure 6: Effect of Antioxidant therapy on glucose levels, HbA1C antioxidant status and sperm DNA fragmentation index (DFI). Evaluation of outcome of antioxidant therapy on glucose levels, HbA1c, antioxidant status, and Sperm DNA fragmentation index (DFI). There are significant improvements in serum glucose levels, MDA, HbA1C and DFI and TAC and SOD in all diabetic patients but more common in those with initial poor glycemic control (P<0.05 to P<0.01) than initial good glycemic control. Surprisingly, there was no difference with glutathione peroxidase.
manifested as double heads, globuzoospermia and macrozoospermia and cytoplasmic mid and tail piece. This is in agreement with an earlier study in which diabetes mellitus of both type I and type II have adverse effects on male sexual and reproductive functions in adolescent boys and men in form of impairment of spermatogenesis, reduced sperm count, serum testosterone and seminal fluid volume, immotility, and loss of libido [24-26]. Diabetic men are certainly at a disadvantage in terms of sperm quality compared with healthy controls [12, 26-28].

The present study has revealed a number of possible factors to explain the phenomenon of how diabetes impacts sperm parameters. About 42.2 to 51.1% of patients in the present study had poor glycemic control. This could be as a result of non-compliance. An equally plausible factor may be insulin resistance which is tied up with low free testosterone [29], as shown in the present study in comparison with the control group. According to Ballester et al. [27] in insulin-dependent diabetes, Leydig cell function and testosterone production decrease because of the absence of the stimulatory effect of insulin on these cells and an insulin-dependent decrease in FSH and LH levels. The significant presence of round and elongated spermatid in semen of men with insulin dependent diabetes found in the present study may be a result of dysregulation of the process of spermatogenesis in which mature spermatozoa are normally released into the adluminal compartment of the seminiferous tubule. The expression and secretion of insulin in human ejaculated spermatozoa has been demonstrated [30], thus providing an autocrine regulation of glucose metabolism according to their energetic needs independent of systemic insulin.

**Oxidative stress and poor glycaemic control: mechanism for sperm damage**

The present study showed a strong association between Diabetes and oxidative stress. The hallmark of poor glycemic control in diabetes mellitus is chronic hyperglycemia, which is directly associated with production and release of free radicals and oxidative stress [31,32] with higher levels of malondialdehyde and reduced antioxidant activity. Glycemic control seems to be a significant factor in the aetiology of abnormality of human sperm (teratozoospermia) and sperm DNA fragmentation. Leukocyte spermia and immature spermatozoa with cytoplasmic mid and tail piece were common findings among diabetic men in the present study. Both enhance Oxidative stress and result in male infertility and developmental abnormalities [25].

**Role for antioxidants**

A significant finding of present study is the improvement of sperm parameters associated with antioxidant therapy; for sperm concentration 47 to 57%, progressive motility 34 to 38%, astheno spermatozoa 44 to 48% and normal sperm morphology 16 to 26%. This shows that antioxidants therapy may improve spermatogenesis including spermatogenesis, intercept and prevent the onslaught of oxidative stress on human spermatozoa, thus prevent impairment of sperm motility, especially in the genital tract. By scavenging of free radical to prevent oxidative stress, antioxidant also prevents sperm DNA damage, as demonstrated in the present study and others [24,33]. It is of clinical interest that combined antioxidant therapy was associated with reduction of blood glucose in poor and good glycemic control, in the present study. This is consistent with studies in which Zinc supplementation in diabetes has been associated with reduction of blood glucose and dyslipidemia [34,35] and antioxidants improved insulin sensitivity, by reducing oxidative stress and insulin resistance [36]. Zinc improves sperm quality on its own as a membrane stabilizer, and as a component of superoxide dismutase prevents sperm apoptosis and sperm DNA fragmentation.

The men with insulin dependent diabetes certainly need tight glycaemic control with insulin. In addition, lifestyle changes in diet rich in natural antioxidants such as vitamins C and E and/or and daily supplementation are advocated. Combining the antioxidants has been advocated because of their different effects on parameters of insulin sensitivity and lipid metabolism [36].

**Conclusion**

Diabetes mellitus has a significant impact on the fertility through impaired sperm quality through oxidative stress. Antioxidant therapy has been shown to significantly improve the sperm quality in men with insulin-dependent diabetes mellitus through reduction of oxidative stress and improvement of the antioxidant status.

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