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Negative Effect of Increased Body Weight on Sperm Conventional and Nonconventional Flow Cytometric Sperm Parameters

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ABSTRACT: Although with some discrepancy, obese men have been reported to have the worst conventional sperm parameters, but little is known about the effects of body weight on sperm mitochondrial function, chromatin condensation and apoptosis. This study was undertaken to evaluate conventional and nonconventional sperm parameters in nonsmoking overweight or obese men without any other cause known to alter sperm parameters. Fifty overweight, 50 obese, and 50 normal-weight healthy nonsmoking men were carefully selected. Each man underwent up to 2 sperm analyses and evaluation of mitochondrial membrane potential (MMP), phosphatidylserine (PS) externalization, chromatin compactness, and DNA fragmentation by flow cytometry. Overweight and obese men had significantly lower sperm progressive motility and normal forms than controls. They also had a significantly higher percentage of spermatozoa with low MMP. Obese, but not overweight, men showed a significantly higher percentage of spermatozoa with PS externalization, an early sign of apoptosis, and a lower percentage of viable spermatozoa. A significant increased percentage of spermatozoa with abnormal chromatin compactness was found in both overweight and obese men, whereas only obese men had a significantly higher number of spermatozoa with DNA fragmentation compared with controls. Healthy nonsmoking overweight and obese men have worse conventional and nonconventional sperm parameters than normal-weight controls. The important role played by these parameters in a couple's fertility suggests a program of body weight loss among the therapeutic repertoire for male infertility.

Key words: Mitochondrial membrane potential, chromatin compactness, sperm apoptosis, DNA fragmentation.

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Statisticians calculate that within 5 years approximately 2.3 million adults will be overweight and 700 million will be obese (World Health Organization [WHO], 2009). Obesity is being regarded as a male factor of infertility, and the data reported so far favor a possible association between increased body weight and sperm parameter abnormalities, although there are conflicting reports (Du Plessis et al, 2010). Several conventional sperm parameters (semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of spermatozoa with normal morphology) have been found worse in obese patients (Jensen et al, 2004; Fejes et al, 2005; Koloszar et al, 2005; Magnusdottir et al, 2005; Nguyen et al, 2007; Hammoud et al, 2008; Stewart et al, 2009; Hofny et al, 2010).

Despite such evidence, it is noteworthy that many overweight and obese men have normal conventional sperm parameters. Accordingly, no effect of obesity has been reported on sperm morphology (Jensen et al, 2004; Magnusdottir et al, 2005). Zorn and colleagues did not find any correlation between the elevated leptin serum levels in obese men and sperm parameters (Zorn et al, 2007). A recent meta-analysis did not report any evidence for a relationship between body mass index (BMI) and sperm concentration or total sperm count. The analysis showed that overweight and obese men have a clearly different sex hormone serum profile, whereas sperm parameter abnormalities are only marginal and below the detection limit of this large study (Aggerholm et al, 2008). Finally, no significant differences in mean BMI and conventional sperm parameters were found in the male partners of couples attending a fertility clinic (Rybar et al, 2011).

Despite the large number of studies on conventional sperm parameters, few authors have explored the relatioship, if any, between body weight and nonconventional sperm parameters. Nonetheless, obesity is a clinical example of systemic oxidative stress (Dandona et al, 2005), and a huge literature has shown the detrimental effects of increased oxidative stress on sperm membrane lipid peroxidation and chromatin and DNA integrity (see Lanzafame et al, 2009 for a review). Accordingly, Kort et al (2006) found a significantly higher DNA fragmentation index in obese

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compared with normal-weight men. High levels of DNA damage have also been reported in obese men compared to normal-weight men (Chavarro et al, 2009). No data have been published on the effects of body weight on mitochondrial function, spermatozoon early apoptosis signs, and chromatin compactness.

Therefore, this study was undertaken to evaluate the effects of increased body weight on conventional (density, motility, and morphology) and nonconventional sperm parameters. To accomplish this, we evaluated sperm mitochondrial function, early signs of apoptosis, chromatin compactness, and DNA fragmentation by flow cytometry in 50 overweight and 50 obese healthy men. The men were carefully selected to avoid the possible interference of cigarette smoking (Calogero et al, 2009) and any andrological disease known to alter conventional and nonconventional sperm parameters. Fifty healthy, nonsmoking, normozoospermic men were selected as a control group.

Materials and Methods

Patient Selection

Fifty overweight (BMI range: 25.1–29.9 kg/m^2), 50 obese (BMI range: 30.1–44.0 kg/m^2), and 50 normal-weight (BMI range: 19.0–24.9 kg/m^2) healthy nonsmoking men were carefully selected for enrollment in this study. All subjects were selected randomly from the general population.

To exclude subjects with the concomitant presence of an andrological disease known to alter conventional and nonconventional sperm paramaters, a complete medical history was collected from each. All men with a negative anamnesis underwent a careful physical examination and laboratory (routine blood testing, sperm analysis, sperm culture, urethral swabs, and sperm analysis) and ultrasound instrumental (didimo-epididymal and prostato-vesicular scans) evaluation.

Men (patients and controls) with systemic and endocrine diseases, sexual dysfunction, irregular sexual activity before sampling (<8 episodes of sexual intercourse in the last month), male accessory gland infection, past or present cryptorchidism or varicocele, microrchidism, cigarette smoke, alcohol or drug abuse, and recent hormonal treatment were excluded. Subjects without any systemic or andrological disease were enrolled in this study, and each underwent blood withdrawal to determine levels of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone, 17ß-estradiol, and sex hormone–binding globulin (SHBG), as well as seminal fluid collection to evaluate conventional and nonconventional sperm parameters.

The protocol was approved by the internal Institutional Review Board, and an informed written consent was obtained from each man.

Sperm Analysis

Two semen samples (7–10 days apart) were collected by masturbation after 4 days of sexual abstinence. After liquefaction, they were analyzed according to the WHO (1999) criteria. The remaining spermatozoa were used for flow cytometry analysis.

Sperm Flow Cytometry Evaluation

Flow cytometry was performed using the flow cytometer EPICS XL (Coulter Electronics, Milan, Italy), as previously reported (Perdichizzi et al, 2007) to evaluate sperm mitochondrial function (after 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride [JC-1] staining), phosphatidylserine (PS) externalization (after annexin V/propidium iodide [PI] double staining), chromatin compactness (after PI staining), and DNA fragmentation using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay.

JC-1 Staining—Mitochondrial membrane potential (MMP) was evaluated by staining with JC-1 (Space Import-Export, Milan, Italy) as previously reported (Perdichizzi et al, 2007). Briefly, the sperm suspension was adjusted to a density of 0.5–1 $\times$ 10^6 cells/mL and incubated with JC-1 for 10–15 minutes at 37°C in the dark.

Annexin V/PI Assay—Staining with annexin V/PI was performed using a commercially available kit (Annexin V-FITC Apoptosis detection kit, Beckman Coulter, Schaumburg, Illinois) as previously reported (Perdichizzi et al, 2007). Briefly, an aliquot containing 0.5 $\times$ 10^6 spermatozoa/mL was resuspended in 0.5 mL of binding buffer, labelled with 1 µL of annexin V-FITC plus 5 µL of PI, incubated for 10 minutes in the dark, and immediately analyzed. Signals were detected through FL-1 (fluorescein isothiocyanate) and FL-3 (PI) detectors. The different labeling patterns in the bivariate PI/annexin V analysis identified different cell populations: annexin negative and PI negative were designated live cells and annexin positive and PI negative as PS-externalized spermatozoa (early apoptotic cells).

PI Staining—Sperm PI staining was performed as previously reported (Perdichizzi et al, 2007). Briefly, semen samples were centrifuged at 500 $\times$ *g* for 10 minutes at room temperature, the supernatant was removed, and spermatozoa were collected. An aliquot of about 1 $\times$ 10^6 spermatozoa was incubated in LPR DNA-Prep reagent containing 0.1% potassium cyanide, 0.1% NaN_3, non-ionic detergents, and salts and stabilized (Beckman Coulter) in the dark, at room temperature for 10 minutes, then incubated in Stein DNA-Prep reagent containing 50 µg/mL of PI (<0.5%), RNAsi type A (4 kunits/mL), <0.1% NaN_3, and salts and stabilized (Beckman Coulter) in the dark, at room temperature for 30 minutes.

TUNEL Assay—TUNEL assay was carried out using the Apoptosis Mebstain kit (Beckman Coulter) as previously reported (Perdichizzi et al, 2007). The negative control was obtained by not adding terminal deoxynucleotidyl transferase to the reaction mixture; the positive control was obtained by pretreating spermatozoa with 1 µg/mL of RNAse-free deoxyribonuclease I (Sigma Chemical, St Louis, Missouri) at 37°C for 60 min before labeling. The debris was eliminated following the same procedure described above.

Table. *Main sperm parameters and hormonal concentrations in normal-weight (BMI range: 19.0–24.9 kg/m^2) healthy men (controls), overweight men (BMI range: 25.1–29.9 kg/m^2), and obese men (BMI range: 30.1–44.0 kg/m^2)*[a]

	Controls (n = 50)	Overweight (n = 50)	Obese (n = 50)
Volume, mL	3.2 ± 0.6	3.3 ± 0.4	3.3 ± 0.8
Sperm density, $\times 10^6$/mL	66.0 ± 5.3	68.2 ± 11.0	57.9 ± 9.7
Total sperm count, $\times 10^6$/ejaculate	211.1 ± 30.2	225.1 ± 44.4	191.7 ± 26.4
Progressive motility, % (a + b)	48.4 ± 4.4	20.2 ± 4.0[b]	23.2 ± 6.0[b]
Normal forms, %	26.2 ± 4.4	22.3 ± 3.4	11.0 ± 2.8[bc]
White blood cells, $\times 10^6$/mL	0.84 ± 0.08	0.66 ± 0.068	0.69 ± 0.37
LH, IU/L	2.7 ± 0.6	3.1 ± 0.8	3.2 ± 0.7
FSH, IU/L	4.4 ± 1.4	4.1 ± 1.2	4.3 ± 1.6
Total testosterone, μg/L	6.6 ± 0.7	6.2 ± 0.5	5.8 ± 0.8
17 β-estradiol, ng/L	21.2 ± 2.3	33.8 ± 3.8[b]	49.8 ± 7.3[b]
SHBG, μmol/L	11.8 ± 1.7	21.2 ± 1.6[b]	23.9 ± 3.3[b]

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone–binding globulin.

[a] Hormonal normal values: LH = 1.7–8.6 IU/L; FSH = 1.5–12.4 IU/L; total testosterone = 2.7–9.6 μg/L; 17β-estradiol = 10–70 ng/L; SHBG = 11–52 μmol/L.

[b] $P < .05$ vs controls.

[c] $P < .05$ vs overweight men.

Statistical Analysis

Results are reported as $\bar{x} \pm$ SEM thoughout the study. Conventional sperm parameters were submitted to statistical analysis as the mean of the 2 determinations obtained from each man enrolled. The data were analyzed by 1-way analysis of variance (ANOVA) followed by the Duncan's multiple range test. The software SPSS 9.0 for Windows was used for statistical evaluation (SPSS Inc, Chicago, Illinois). A statistically significant difference was accepted at $P < .05$.

RESULTS

The age of overweight (31.2 ± 1.2 years, range: 20–43 years) and obese (31.6 ± 1.7 years (range: 22–48 years) men did not differ significantly from that of controls (31.5 ± 1.1 years, range: 22–46 years).

Among overweight patients, 70% (n = 35) showed a BMI between 27.0 and 28.5 kg/m^2, whereas among obese patients, 80% (n = 40) showed a BMI between 37.5 and 39.5 kg/m^2 (class II; WHO, 1995). Finally, among controls 70% showed a BMI between 21.5 and 23.5 kg/m^2.

Conventional sperm parameters and hormonal serum levels are reported in the Table. Both overweight and obese men had a significantly lower spermatozoa with progressive motility (grade a + b) compared with controls ($P < .05$, ANOVA followed by Duncan's test), whereas only obese men had a significantly lower percentage of normally shaped spermatozoa compared with both controls and overweight men ($P < .05$, ANOVA followed by Duncan's test). Seminal fluid volume, sperm density, sperm total count, and seminal white blood cells did not show any significant variation in either overweight or obese men.

Serum LH, FSH, and total testosterone levels did not differ significantly, whereas 17ß-estradiol and SHBG were significantly higher in both overweight and obese men compared with controls ($P < .05$ vs controls, ANOVA followed by Duncan's test).

Both overweight and obese men had a significantly higher percentage of spermatozoa with low MMP compared with controls ($P < .05$, ANOVA followed by Duncan's test). The percentage of spermatozoa with low MMP in obese men was significantly higher compared with overweight men ($P < .05$, ANOVA followed by Duncan's test; Figure 1). The percentage of spermatozoa with PS externalization was higher in both overweight and obese men, but only in the latter did the difference reach statistical significance ($P < .05$, ANOVA followed by Duncan's test; Figure 2, upper panel). Likewise, the

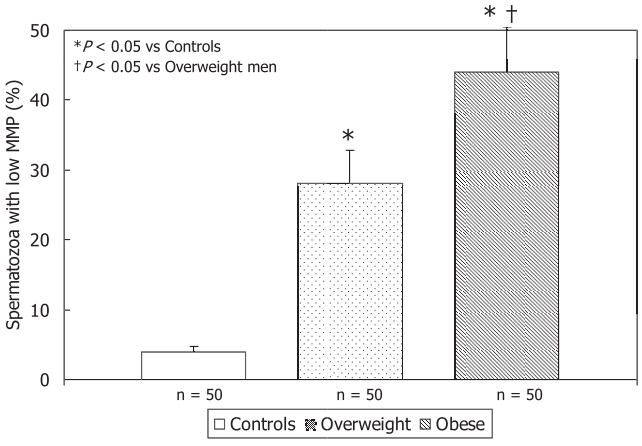

Figure 1. Percentage of spermatozoa with low mitochondrial membrane potential (MMP) in normal-weight (body mass index [BMI] range: 19.0–24.9 kg/m^2) healthy men (controls), overweight men (BMI range: 25.1–29.9 kg/m^2), and obese men (BMI range: 30.1–44.0 kg/m^2).

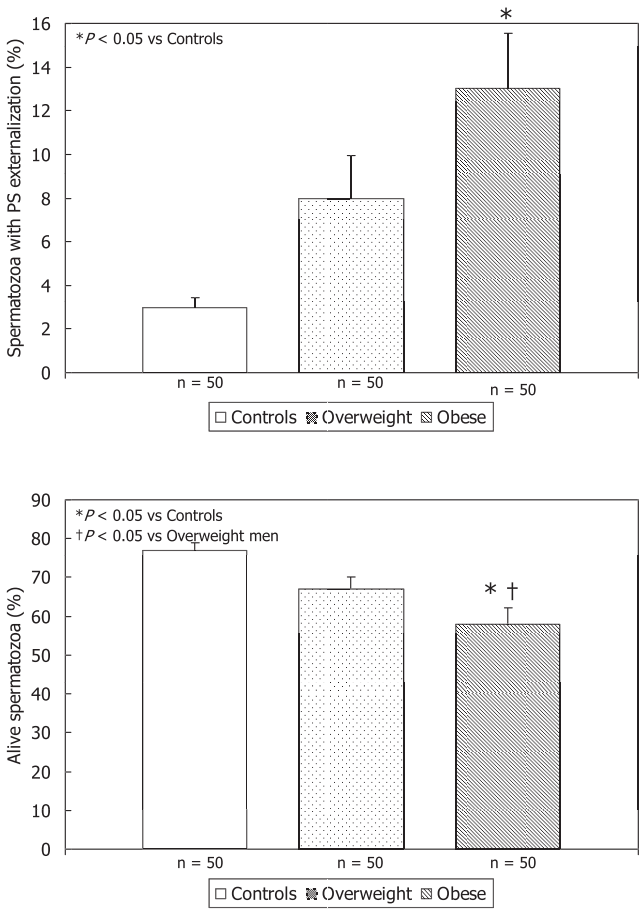

Figure 2. Percentage of spermatozoa with externalization of phosphatidylserine (PS; **upper panel**) or live (annexin- and propidium iodide–negative cells; **lower panel**) in normal-weight (body mass index [BMI] range: 19.0–24.9 kg/m^2) healthy men (controls), overweight men (BMI range: 25.1–29.9 kg/m^2), and obese men (BMI range: 30.1–44.0 kg/m^2).

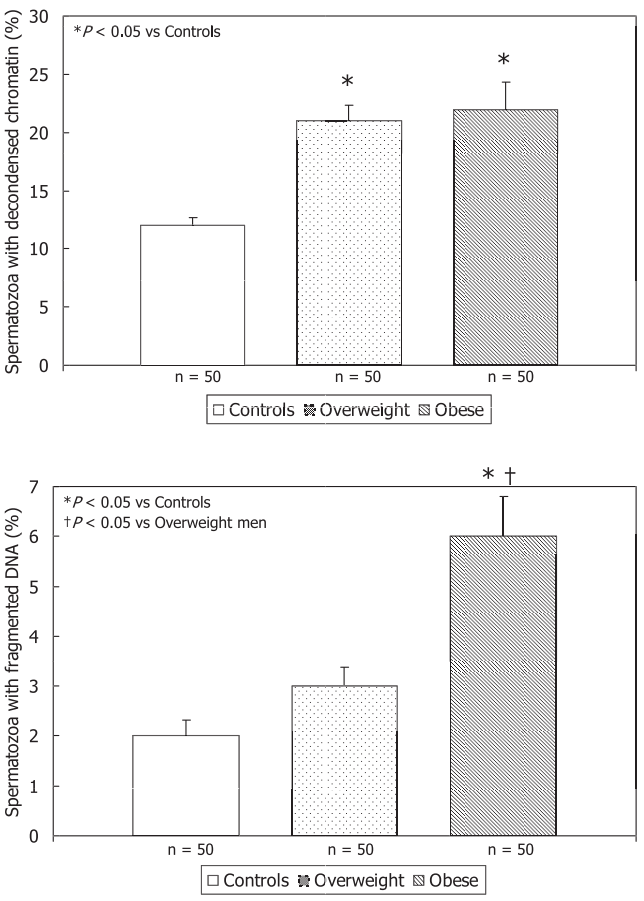

Figure 3. Percentage of spermatozoa with decondensed chromatin (**upper panel**) or DNA fragmentation (**lower panel**) in normal-weight (body mass index [BMI] range: 19.0–24.9 kg/m^2) healthy men (controls), overweight men (BMI range: 25.1–29.9 kg/m^2), and obese men (BMI range: 30.1–44.0 kg/m^2).

percentage of live spermatozoa, evaluated by annexin V/PI assay and flow cytometry, decreased significantly only in obese men compared with both controls and overweight men ($P < .05$, ANOVA followed by Duncan's test; Figure 2, lower panel). The percentage of spermatozoa with decondensed chromatin was significantly higher in both overweight and obese men compared with controls ($P < .05$, ANOVA followed by Duncan's test; Figure 3, upper panel), whereas the percentage of spermatozoa with fragmented DNA was significantly higher only in obese men compared with controls and overweight men ($P < .05$, ANOVA followed by Duncan's test; Figure 3, lower panel).

DISCUSSION

In the present study, we examined sperm quality in overweight and obese men who did not have any identifiable systemic or andrological cause of sperm parameter abnormality after a thorough clinical, laboratory, and instrumental evaluation. The results confirmed that overweight and, to a greater extent, obese men have progressively lower motile spermatozoa and normal forms compared with healthy normal-weight men. In addition, we showed for the first time that overweght and obese men had an increased percentage of spermatozoa with low MMP and PS externalization, an early sign of apoptosis. The percentage of spermatozoa with fragmented DNA was significantly higher only in obese men.

Several conventional sperm parameters (semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of spermatozoa with normal morphology) have been found to correlate inversely with BMI in a large sample of Danish military recruits, with less favorable values observed in men with a BMI either less than 20 kg/m^2 or greater than 25 kg/m^2 (Jensen et al, 2004). A study investigating factors

associated with poor semen quality among couples who visited an assisted reproduction clinic showed that the prevalence of obesity among the male partners was 3 times higher than that of men of couples with idiopathic or female factor infertility (Magnusdottir et al, 2005). Koloszar et al (2005) estimated that sperm concentration and total sperm count among men with BMI $>$ 25 kg/m^2 were 26.1% and 23.9% lower, respectively, than in men with a BMI ranging from 20 to 25 kg/m^2. The frequency of oligozoospermia was 29% among overweight men compared with 21.7% in the normal-weight reference group (Fejes et al, 2005). A retrospective cohort study, including more than 26 000 pregnancies, reported an odds ratio for infertility of 1.2 for overweight and 1.36 for obese men compared with men with low to normal BMI (Nguyen et al, 2007). Hammoud et al (2008) reported that the incidence of oligozoospermia and asthenozoospermia increased with augmentation of BMI. In another recent study, 35 obese subjects had significantly lower total sperm count compared with 188 men with a BMI of $<$30 kg/m^2 (Stewart et al, 2009). Additionally, BMI correlates positively with abnormal sperm morphology and negatively with sperm concentration and motility in obese fertile and infertile men (Hofny et al, 2010).

Although many studies have explored the effect of body weight on conventional sperm parameters (Jensen et al, 2004; Fejes et al, 2005; Koloszar et al, 2005; Magnusdottir et al, 2005; Aggerholm et al, 2008; Hammoud et al, 2008; Stewart et al, 2009), few have examined the effects on nonconventional sperm parameters (Kort et al, 2006; Chavarro et al, 2009). By sperm chormatin structure assay, the DNA fragmentation index (DFI) was significantly higher in overweight and obese men compared with normal-weight men. No significant difference was found between the overweight and obese groups. A linear regression analysis revealed a significant and positive relationship between BMI and DFI (Kort et al, 2006). While we were conducting the present study, Chavarro et al (2009) reported that obese men had a higher number of spermatozoa with DNA damage, evaluated by COMET assay, than normal-weight men. The results of these studies are similar to the results of the present study which, although it included a smaller number of patients, extended the evaluation of the effects of increased body weight on sperm mithocondrial function; PS externalization, an early sign of apoptosis; and chromatin integrity. Another important feature of this study is the strict selection of the patients enrolled. Indeed, after a through diagnostic work-up, only overweight and obese men without any identifiable known cause of sperm parameter abnormality were enrolled. This careful selection suggests a possible cause and effect relationship between BMI and the conventional and nonconventional sperm parameters evaluated.

The sperm biofunctional parameters evaluated in this study are important indicators of sperm quality (Zini and Libman, 2006) and, in particular, sperm DNA integrity (Agarwal and Said, 2005). Evidence indicates that only a small percentage of spermatozoa in normal men have fragmented DNA (Cohen-Bacrie et al, 2009). Three main theories attempt to explain the mechanisms that generate these breaks: 1) incomplete maturation during spermiogenesis, 2) oxidative stress, and 3) uncontrolled apoptosis (Sakkas and Alvarez, 2010). The increased sperm DNA fragmentation that we and others (Kort et al, 2006; Chavarro et al, 2009) found in obese men suggests that obesity could be a pro-apoptotic condition. This hypothesis is supported by the contention that overweight men have a similar percentage of spermatozoa with DNA fragmentation as normal-weight men, whereas they have an increased number of spermatozoa with low MMP or PS externalization. The alteration of MMP anticipates the exposure of PS on the external leaflet of the plasma membrane, a potentially reversible signal, which starts the cascade of events leading to final DNA fragmentation (Zhang et al, 2008). Possible triggering mechanisms are oxidative stress and an altered hormonal control (Coviello et al, 2005; Dandona et al, 2005; Pauli et al, 2008; Sakkas and Alvarez, 2010). The accumulation of oxygen free radicals (Bakos et al, 2010), the high rate of circulating estrogen (Kley et al, 1980), and the low levels of gonadotropins (Pauli et al, 2008) could be potential treatment targets.

Healthy overweight and obese men have worse sperm progressive motility and morphology and nonconventional sperm parameters, including DNA and chromatin integrity and, for the first time, mitochondrial function, than normal-weight men. Given the relevant role played by these sperm parameter abnormalities in a couple's fertility, we suggest including a program of body weight loss among the therapeutic strategies for male infertility.

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