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ORIGINAL ARTICLE

Effects of leptin on sperm count and morphology in Sprague-Dawley rats and their reversibility following a 6-week recovery period

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Summary

Altered epididymal sperm count and morphology following leptin treatment has been reported recently. This study examined the effects of 42 days of leptin treatment on sperm count and morphology and their reversibility during a subsequent 56-day recovery period. Twelve-week-old male Sprague-Dawley rats were randomised into four leptin and four saline-treated control groups ($n = 6$). Intraperitoneal injections of leptin were given daily (60 μg Kg^{-1} body weight) for 42 days. Controls received 0.1 ml of 0.9% saline. Leptin-treated animals and their respective age-matched controls were euthanised on either day 1, 21, 42 or 56 of recovery for collection of epididymal spermatozoa. Sperm concentration was determined using a Makler counting chamber. Spermatozoa were analysed for 8-hydroxy-2-deoxyguanosine and DNA fragmentation (Comet assay). Data were analysed using ANOVA. Sperm concentration was significantly lower but fraction of abnormal spermatozoa, and levels of 8-hydroxy-2-deoxyguanosine were significantly higher in leptin-treated rats on day 1 of recovery. Comet assays revealed significant DNA fragmentation in leptin-treated rats. These differences were reduced by day 56 of recovery. It appears that 42 days of leptin treatment to Sprague-Dawley rats has significant adverse effects on sperm count and morphology that reverse following discontinuation of leptin treatment.

Introduction

Leptin has been shown to have roles in numerous physiological processes including regulation of body weight, food intake, cardiovascular function, immune function, hematopoiesis, inflammation, sexual maturation and normal reproduction (Fantuzzi & Faggioni, 2000; Haluzik *et al.*, 2000; Mantzoros, 2000; Waelput *et al.*, 2006; Sayed-Ahmed *et al.*, 2012). With regard to reproduction, treatment of infertile *ob/ob* mice with leptin restores their reproductive ability (Barash *et al.*, 1996; Mounzih *et al.*, 1997). Leptin receptor isoforms have been reported in gonadal tissue, suggesting a direct endocrine action of leptin on the gonads (Zamorano *et al.*, 1997; El-Hefnawy *et al.*, 2000). The precise role of leptin in the testes has not been fully established and remains a focus of current studies. The presence of leptin receptors in Leydig and Sertoli cells suggests that it might have a role in the endocrine function of the testes and in spermatogenesis. Interestingly, leptin is secreted by human ejaculated spermatozoa, and the secretion is significantly higher in spermatozoa incubated in capacitating conditions (Aquila *et al.*, 2005). Leptin together with insulin has also been shown to enhance human sperm motility (Lampiao & du Plessis, 2008).

Whilst the role of leptin in normal reproductive function is now increasingly recognised, leptin administration to normal adult non-obese rats, has been found to adversely affect sperm count and sperm morphology (Haron *et al.*, 2010, 2013; Abbasihormozi *et al.*, 2013). Whilst the link between obesity related infertility and leptin remains to be established; however, it remains unknown if the reported effects of exogenous leptin administration on sperm count and morphology are reversible upon discontinuation of leptin treatment. Besides, the duration required for recovery is also not

known. This study therefore examined the effect of 21, 42 and 56-day recovery period following 42 days of leptin treatment on sperm count and morphology in normal adult rats. In addition, it also examined evidence for DNA damage following leptin treatment and recovery.

Materials and methods

Twelve-week-old male Sprague-Dawley rats were randomised into four leptin-treated and four saline-treated, age-matched control groups with six rats per group. Intraperitoneal injections of leptin at a dose of 60 μg kg^{-1} body weight in 0.1 ml 0.9% saline were given daily for 42 days (recombinant rat leptin, purity >95%; Biovision, San Francisco, USA). Controls received 0.1 ml of 0.9% saline over the same period. The duration and dose of leptin treatment were based on our previous studies in the rat where we found that a more pronounced effect of leptin was evident following 42 days of treatment than after 7 or 21 days of treatment (Haron *et al.*, 2010, 2013). Although the dose of leptin used in these studies ranged from 5–30 μg Kg^{-1}, but to ensure a more pronounced effect, a dose of 60 μg Kg^{-1} was used in this study. On day 43, that is 1 day after the completion of leptin treatment, one group of leptin-treated rats together with an age-matched saline-treated control group was euthanised for collection of epididymal spermatozoa. This provided the sperm data for day 1 of the recovery period. The remaining three groups, together with their age-matched saline-treated controls, were allowed to recover further and then euthanised at either days 21, 42 or 56. Euthanisation was carried out using a small animal guillotine. The duration of the recovery period was approximated to the 54-day sperm cycle in the rat. Days 21 and 42 were chosen arbitrarily as representing 35–40 and 70–75%, respectively the length of the sperm cycle. The study design was approved by the Animal Care and Users Committee, Universiti Teknologi MARA.

Sperm collection

Immediately following euthanisation, a laparotomy was performed, and the left epididymis was removed and weighed. The cauda of each epididymis was cut and minced separately in 2 ml of normal saline and filtered through nylon mesh for collection of spermatozoa (Haron *et al.*, 2010). An aliquot of the epididymal suspension was then used for sperm count and morphology using a Makler counting chamber (Sefi Medical Instruments LTD, Haifa, Israel). The remainder was used for sperm DNA damage detection and estimation using comet assay and sperm DNA damage marker 8-hydroxy-2-deoxyguanosine (8-OHdG) respectively.

Sperm concentration and morphology

The sperm specimen was mixed thoroughly, and a small drop was then pipetted onto the centre of the disc area of a Makler chamber. The number of spermatozoa in a strip of 10 squares was counted. This number represented the concentration in million per ml. This was repeated on another strip, and the average of the two counts was recorded. The number of abnormal spermatozoa in the same strip of 10 squares was counted and recorded. Spermatozoa were classified into normal and abnormal types as described previously (Narayana *et al.*, 2002). In brief, the spermatozoa with the following characteristics were considered abnormal: (i) headless, (ii) hookless, (iii) cephalo-cauda, (iv) double headed, (v) broken tail, (vi) microcephalic, (vii) coiled tail, (viii) double tailed.

Quantitative measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG)

Genomic DNA was isolated from spermatozoa sample of each animal using Genomic DNA Isolation Kit (Biovision USA). The isolated DNA was first hydrolysed into nucleotides by incubating it with 100 U ml^{-1} nuclease P1 (Sigma-Aldrich, St Louis, USA) in 20 mM sodium acetate buffer (pH 4.5) at 37 °C for 60 min. Following this, one unit of alkaline phosphatase (Sigma-Aldrich) was added per 100 μg of DNA and incubated at 37 °C for another 30 min. After incubation, the DNA lysate was boiled for 10 min and then placed on ice until measurement. The level of 8-OHdG was estimated using 8-hydroxy-2-deoxy Guanosine EIA Kit (Cayman Chemical, Michigan, USA).

Detection of sperm DNA damage using comet assay

Sperm DNA damage was assessed using the single cell gel/comet assay according to Tripathi & Jena (2008). Fully frosted slides were covered with 100 μl of 1% normal melting agarose (NMA), and with a cover slip in place, the agarose was allowed to solidify at 4 °C for 5 min. An agarose sperm suspension was prepared by mixing 10 μl of fresh sperm suspension containing 1×10^5 spermatozoa with 90 μl of 1% low melting agarose (LMA).

After removal of the cover slip from the frosted slides, 85 μl of the sperm suspension in 1% LMA was pipetted onto the slides to form the second layer. A fresh cover slip was placed, and the agarose was allowed to set at 4 °C for 10 min. The cover slips were then removed, and the slides were placed in jars containing lysis buffer (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris containing 1% Triton X-100, 40 mM DL- dithiothreitol and proteinase K 0.5 mg/ml) at 37 °C for 24 h. Following lyses, all

slides were washed three times with deionised water at 10 min interval to remove salt and detergent from the gels. Slides were then coded and placed in a horizontal electrophoresis tank (Model Sub-Cell GT Basic, BIO-RAD, Hercules, CA, USA, Max. volts 1000 V, Max. current 500 mA) filled with fresh alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA pH 13) for 20 min to allow the DNA from the cells to unwind. Electrophoresis was performed at room temperature and conducted at 25 V, 300 mA for 20 min. The slides were then washed and neutralised using neutralisation buffer (0.4 M Tris-Base, pH 7.5) in three different jars with the duration of wash at 5 min per jar. After neutralisation, the slides were dried and stained with DNA SYBR Green 1 (1 : 10 000 dilution) for 30 min. The slides were then rinsed quickly with double-distilled water, and cover slips were placed for image analysis. The fluorescent labelled DNA was visualised (200×) using a BX 61 Imager fluorescence microscope (Olympus Co., Tokyo, Japan), and the resulting images were captured on a computer. The analyses of comet images were performed by the automated software Comet Assay Software Project lab V.1.2.3b1 (CASP, Sourceforge, Diceholdings Inc, NY, USA). Samples were run in duplicate, and 100 cells were randomly analysed per slide for a total of 200 cells per sample and scored for tail length (TL), tail moment (TM), olive tail moment (OTM) and % tail DNA (% DNA).

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) for multiple comparisons followed by *post hoc* Tukey's analysis contained in the SPSS version 20, IBM, NY, USA and expressed as mean ± SEM. Statistical significance was accepted at $P < 0.05$. No animals were lost during the study, and data from all the rats are included in the analysis.

Results

Body weight increased significantly over the duration of the study in both groups ($P < 0.001$), but there were no significant differences in body weight between leptin-treated rats and their age-matched controls either during leptin treatment or during the recovery period (Table 1).

Mean sperm count at days 1, 21 and 42 of recovery was significantly lower in leptin-treated rats when compared to that in age-matched controls ($P < 0.001$, 0.001 and 0.05, respectively; Fig. 1). Total sperm count increased during recovery, and no significant difference was evident in sperm count between leptin-treated rats and their respective age-matched controls on day 56 of recovery.

The fraction of spermatozoa with abnormal morphology at days 1, 21 and 42 of recovery was also significantly higher in leptin-treated rats when compared to that in the controls ($P < 0.001$, 0.001 and 0.01, respectively; Fig. 2). Headless and coiled-tail spermatozoa were the two most common types of abnormal sperm seen. The fraction of spermatozoa with abnormal morphology, however, declined with increasing duration of recovery, where, by day 56 of recovery no significant difference was evident between the two groups.

Sperm 8-OHdG levels were significantly higher immediately after completion of 42 days of leptin treatment, that is at recovery day 1 (Fig. 3). Its concentration, however, declined over the recovery period, and the difference was no longer significant from that in non-treated age-matched controls at day 56 of recovery.

Compared to the controls, comet assay revealed significantly higher tail length, tail moment, olive tail moment and % tail DNA in leptin-treated rats both immediately after the completion of leptin treatment (i.e. recovery day 1) and at recovery days 21, 42 and 56 (Table 2; Fig. 4). There was, however, a general tendency for all these parameters to shift towards the control values with increasing duration of recovery.

Discussion

The major findings of this study include (i) a significantly lower sperm count following leptin treatment that returned to levels similar to those in the controls by day 56 following the discontinuation of leptin treatment, (ii) significantly higher fraction of spermatozoa with

Table 1 Body weight in control and leptin-treated rats

Day	1	21	42	56
Leptin treated	Leptin treatment		Recovery period	
	413.6 ± 6.4 (n = 6)	418.5 ± 5.4 (n = 6)	464.8 ± 4.6### (n = 6)	525.3 ± 2.75### (n = 6)
Control saline	Saline treatment		Recovery period	
	412.6 ± 6.32 (n = 6)	419.7 ± 3.86 (n = 6)	489.2 ± 7.07### (n = 6)	514.0 ± 1.99###(n = 6)

###$P < 0.001$ compared to their respective weight at day 0 (i.e. day 43 or one day after the completion of leptin treatment).

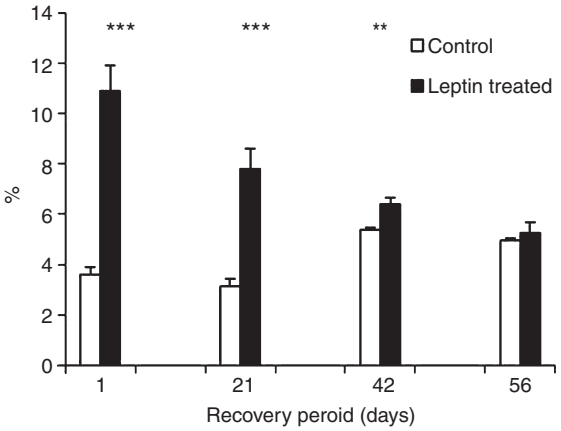

Fig. 1 Sperm morphology in controls and leptin-treated rats during the recovery period. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to their respective controls.

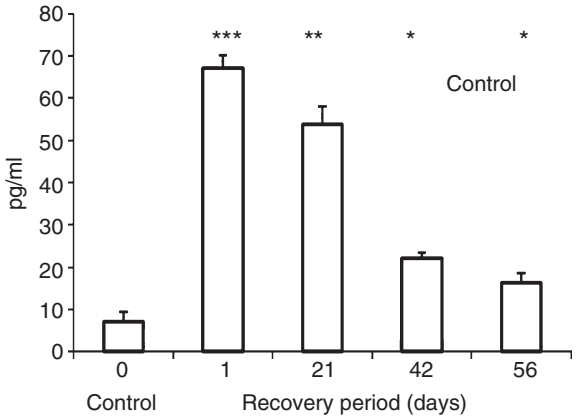

Fig. 3 Sperm 8-hydroxy-2-deoxyguanosine (8-OHdG) levels in leptin-treated rats during recovery and their age-matched controls. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$.

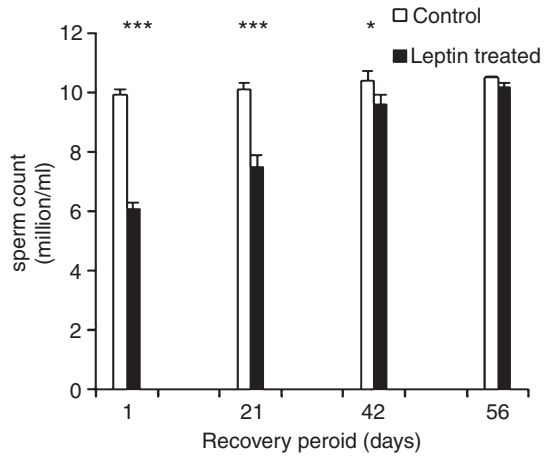

Fig. 2 Sperm count in leptin-treated and control rats during the recovery period. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to their respective controls.

abnormal morphology following leptin treatment, which recovered to levels similar to those in the controls by day 56 of recovery, (iii) significantly higher DNA fragmentation that did not completely recover by day 56 following the discontinuation of leptin treatment, and (iv) increased levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) levels following leptin treatment that returned to levels similar to those in the controls by day 56 of recovery.

Decreases in sperm count and increased fraction of spermatozoa with abnormal morphology following leptin treatment in the rat have been reported before (Haron *et al.*, 2010, 2013; Abbasihormozi *et al.*, 2013). Increased serum FSH and LH levels following leptin administration were also reported in these studies. Although leptin has been shown to influence the release of gonadotrophin-releasing hormone (Quennell *et al.*, 2009), it is unlikely that the lower sperm count and the increased fraction of morphologically abnormal spermatozoa following leptin treatment were due to the effect of leptin on the pituitary-gonadal axis. Serum testosterone levels were not significantly altered following leptin treatment (Haron *et al.*, 2010). A direct effect of leptin on the testes and on spermatogenesis, perhaps involving oxidative stress, therefore appears more likely. In this regard, increased production of reactive oxygen species (ROS) and markers of oxidative stress in the testes following leptin treatment have been reported recently (Abbasihormozi *et al.*, 2013). In addition, leptin has also been shown to cause peroxynitrite-mediated oxidative stress in steatohepatic lesions (Chatterjee *et al.*, 2013) and correlate negatively with paraoxonase activity in venous blood of obese pregnant women (Ferretti *et al.*, 2013). Moreover, a negative correlation between ROS levels and semen quality has also been reported (Gomez *et al.*, 1998; Agarwal *et al.*, 2003, 2005; Makker *et al.*, 2009). Although low levels of ROS activity are considered necessary for normal sperm function, higher levels could be detrimental and could increase DNA fragmentation and apoptosis in the spermatozoa and the seminiferous tubules. Comet assay analysis revealed evidence of significant DNA fragmentation following leptin treatment, which decreased with increasing duration of recovery (Table 2; Fig. 4). In addition, sperm 8-OHdG, a useful marker for the assessment of the impact of oxidative stress on DNA, was significantly higher in leptin-treated rats, particularly immediately after the 42-day treatment (Fig. 3). The precise mechanism responsible for the increased oxidative

Table 2 Comet assay summary sheet during the recovery periods

Groups	Tail length (n = 6)	Tail moment (n = 6)	Olive tail moment (n = 6)	%Tail DNA (n = 6)
Control	19.25 ± 2.9	2.09 ± 0.43	2.25 ± 0.56	0.88 ± 0.09
Leptin (Recovery day 1)	39.87 ± 0.9***	7.19 ± 0.3***	6.56 ± 0.14***	1.61 ± 0.07***
Leptin (Recovery day 21)	35.57 ± 1.08***	6.01 ± 0.27***	6.14 ± 0.2***	1.37 ± 0.1***
Leptin (Recovery day 42)	34.59 ± 1.7***	5.9 ± 0.51***	5.69 ± 0.34***	1.29 ± 0.13**
Leptin (Recovery day 56)	27.55 ± 1.56**	4.09 ± 0.37**	4.61 ± 0.31**	1.19 ± 0.11*

*P < 0.05; **P < 0.01 and ***P < 0.001 compared to the control.

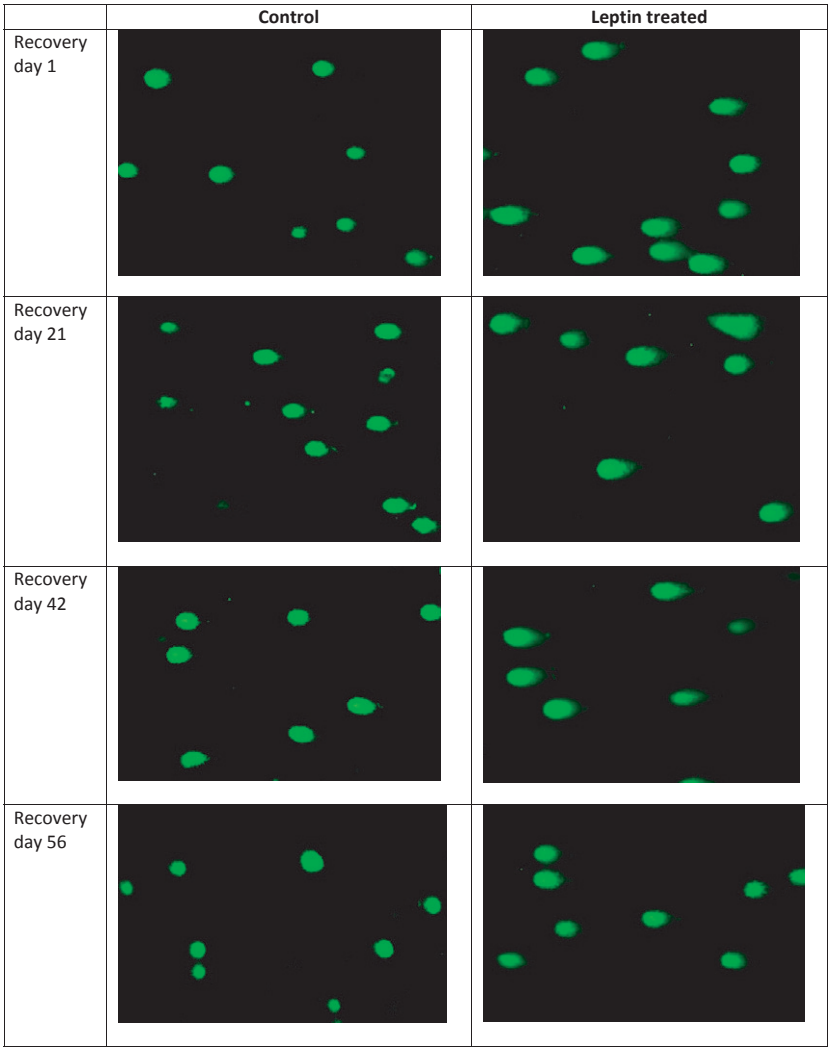

Fig. 4 Photomicrographs showing DNA migration pattern of sperm nuclei in single cell gel electrophoresis (comet assay) stained with SYBR Green-I during recovery.

stress following leptin treatment, however, remains unclear. DNA fragmentation can arise from numerous intrinsic and extrinsic factors. As sperm collection, storage and handling techniques were the same in both the control and leptin-treated groups in this study, it would therefore be reasonable to assume that the differences in the levels of DNA fragmentation evident in this study are probably due to intrinsic causes secondary to leptin treatment. Whilst the increased levels of 8-OHdG in leptin-treated rats suggest a role for oxidative stress in the DNA fragmentation, there is still a need to ascertain whether the mechanism of the increased fragmentation involves other sources as well. It could result from deficiencies in recombination during spermatogenesis, abnor-

mal spermatid maturation or even variations in sperm protamine expression during spermiogenesis. It is known that during spermiogenesis, 85–95% of histones in the DNA are replaced by protamines 1 and 2 (P1, P2) resulting in compaction of the nucleus and cessation of gene expression (Conwell *et al.*, 2003; Oliva, 2006; Barratt *et al.*, 2010). Altered P1/P2 ratios have been associated with increased DNA fragmentation (Garcia-Peiro *et al.*, 2011; Simon *et al.*, 2011). The impact of leptin on spermatogenesis has not been reported before, and it is possible that leptin either directly or through increased oxidative stress might be influencing it, resulting in increased DNA fragmentation. The pattern of changes in DNA fragmentation and sperm 8-OHdG levels seems to suggest the involvement of oxidative stress (Fig. 3). Studies involving the use of anti-oxidants could help identify the role of oxidative stress in leptin-induced increased DNA fragmentation. Preliminary studies in our laboratory using anti-oxidants seem to implicate the role of oxidants in leptin-induced changes in sperm count and morphology (FA Almabhouh, HJ Singh, unpublished data).

The finding of adverse effects of leptin on spermatozoa might have relevance in obesity related infertility in males. The prevalence of infertility, for example, is reportedly high in obese males (Nguyen *et al.*, 2007). In addition, a recently published review of 23 studies on sperm function found 15 of these studies reporting a lower sperm concentration in obese males (Palmer *et al.*, 2012). Serum leptin concentrations are positively correlated with total body fat mass (Shimizu *et al.*, 1997), and it is possible the high levels of leptin might be responsible for the lower sperm concentration in obese males.

The fraction of spermatozoa with abnormal morphology seems to increase slightly with age in controls (Fig. 2). Age-related decrease in sperm quality has been reported before, but the precise reason for this remains unclear although it has been attributed to age-related degeneration or senescence in the seminiferous tubules, seminal vesicle and the prostate (Kidd *et al.*, 2001; Eskenazi *et al.*, 2003). Body weight was not different between the controls and leptin-treated groups during leptin treatment or during recovery (Table 1). The lack of effect on body weight following leptin treatment could be due to the dose used and to the fact that it was given as a single daily dose. Given its short half-life of 9–12 min (Zeng *et al.*, 1997) it might be argued that its effects on appetite might only be transient, and a rebound feeding could prevent any changes in body weight. Suppression of food intake for a short duration is often also associated with a rebound increase in food intake for a short while afterwards (Kumar *et al.*, 2002). Of more importance in this study is that the effects of leptin on spermatozoa were evident without significant differences in the changes in body weight.

Whilst the effect of leptin on sperm morphology and count has been reported before, to date no reports exist as to whether these effects are reversible following cessation of leptin treatment. Data from this study suggest that discontinuation of leptin after 42 days of treatment at a dose of 60 μg kg^{-1} requires an approximate duration of at least 56 days or more for recovery from leptin-induced changes in sperm count and morphology (Figs 1 and 2).

The requirement of up to 56 days or more for a significant recovery from the adverse effects of leptin seems to suggest that the adverse effects of leptin might include its affects on processes further upstream in spermatogenesis. The further upstream the effect, the longer will be the duration required for complete recovery. The upstream effect could involve the early phases of spermatogenesis. Spermatogenesis can be described to occur in three phases. The first is where the spermatogonia undergo a series of mitotic amplifying divisions and differentiate into spermatocytes. The second phase is where the spermatocytes undergo meitotic recombination giving rise to haploid spermatids. The third, termed spermiogenesis involves transformation into mature spermatozoa involving chromatic remodelling and replacement of histones with transition proteins first and then with protamines. Leptin, it seems, could influence any one or all of these processes. Clearly, more studies are needed to investigate this.

In conclusion, it appears that exogenous leptin administration adversely affects sperm count and morphology. It also significantly increases DNA fragmentation and the DNA marker of oxidative stress. These effects are reversible following the discontinuation of leptin treatment and require about 8 weeks.

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