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Research Article

Effects of Profortil® on Leptin-Induced Adverse Effects on Sperm Parameters in Sprague-Dawley Rats

Ifrah Alam Malik (iD),[1] **Damayanthi Durairajanayagam** (iD),[1] **Ralf Henkel** (iD),[2,3,4] **and Harbindar Jeet Singh** (iD)[1,5]

[1]Department of Physiology, Faculty of Medicine, Jalan Hospital, Universiti Teknologi MARA, Sungai Buloh 47000, Selangor, Malaysia
[2]Department of Medical Bioscience, University of the Western Cape, Robert Sobukwe Road, Bellville 7535, South Africa
[3]Department of Metabolism, Digestion and Reproduction, Imperial College London, South Kensington Campus, London SW7 2AZ, UK
[4]LogixX Pharma, Brunel Road, Theale RG7 4AB, Berkshire, UK
[5]I-PPerForM, Faculty of Medicine, Jalan Hospital, Universiti Teknologi MARA, Sungai Buloh 47000, Selangor, Malaysia

Correspondence should be addressed to Damayanthi Durairajanayagam; damayanthi.d@gmail.com

Raised leptin levels induce oxidative stress, which negatively impacts male reproduction. Profortil® is an antioxidant formulation clinically used to improve sperm quality. Whether Profortil® supplementation can prevent the detrimental effects of leptin on spermatozoa is not known. Thus, the aim of this study was to examine the effects of Profortil® on leptin-induced adverse effects on rat spermatozoa. Adult Sprague-Dawley rats were given either normal saline (control), leptin ($60\,\mu g$/kg/day), Profortil® (50 mg/kg/day), or Profortil® + leptin once daily for 2 weeks. Sperm count and abnormal morphology were recorded. Testicular levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), superoxide dismutase (SOD), and catalase (CAT) activities were measured. TUNEL and Comet assays were performed. The sperm count in the Profortil® + leptin group was comparable to that in controls but was significantly higher than that in the leptin group, which was significantly lower than that in controls. Sperm morphology was not different between leptin and Profortil® + leptin groups. 8-OHdG level was significantly lower in the Profortil® + leptin group than that in the leptin group but was comparable to that in controls. CAT activity was not different between leptin and Profortil® + leptin groups, although activity in the former was significantly lower than that in controls. SOD activity was not different between groups. The apoptotic index was significantly higher in the leptin and Profortil® + leptin groups than in the control and Profortil® groups. Sperm DNA fragmentation was not significantly different between leptin and Profortil® + leptin groups. In conclusion, Profortil®, when given at 50 mg/kg/day for 3 weeks, was only able to reduce some, but not all the adverse effects of leptin on sperm parameters.

1. Introduction

Profortil® (Lenus Pharma, Vienna, Austria), a concoction of several different antioxidants, is used clinically to improve sperm quality in humans. It consists of L-carnitine, L-arginine, vitamins E and B9 (folic acid), coenzyme Q10 (CoQ10), glutathione, zinc, and selenium (Table 1). Several studies have reported improved sperm motility and morphology [1, 2], and live birth rates [2–4] following Profortil® treatment in infertile couples.

Like Profortil®, other antioxidant formulations made up of multivitamins and minerals have been used as part of the treatment of male infertility, albeit, with mixed results. Menevit® and Androferti are two formulations that have been previously tested in infertile males resulting in improved sperm integrity [5, 6]. Fertilix® administration to GPx5 knock-out mice led to an improvement in oxidative damage in sperm DNA [7]. However, these antioxidant formulations have yet to be examined if they can decrease or prevent leptin-induced adverse effects on spermatozoa, except for melatonin. Concurrent administration of melatonin, a potent antioxidant, was found to prevent the adverse effects of leptin on rat spermatozoa [8].

Leptin is an adipokine produced primarily by white adipose tissue, and its concentration in plasma correlates positively with body fat mass percentage [9, 10]. Raised leptin levels are implicated in several diseases associated with obesity, including male infertility [11, 12]. Administration of exogenous leptin to adult, normal weight male Sprague-Dawley rats decreases total sperm count, and increases the fraction of sperm with abnormal morphology, sperm DNA fragmentation, levels of reactive oxygen species, and 8-hydroxy-2-deoxyguanosine (8-OHdG) [8, 13–17]. The effects of leptin on spermatozoa are a consequence of oxidative stress, most likely involving the PI3K pathway [17, 18].

The adverse effects of leptin on sperm are caused by increased oxidative stress, and these effects are preventable by antioxidants such as melatonin [19]. However, it is unclear if the combination of antioxidants in Profortil® could similarly prevent or ameliorate the effects of leptin on spermatozoa. Thus, this study investigated the effects of Profortil® on the adverse effects induced by leptin on sperm parameters in adult Sprague-Dawley rats.

2. Materials and Methods

2.1. Animals. Twelve to fourteen weeks old male Sprague-Dawley rats, weighing 350–400 g, were acquired from the Laboratory Animal Care Unit (LACU), Universiti Teknologi MARA (Sungai Buloh, Selangor, Malaysia). Each rat was kept in a separate cage at room temperature ($22-25°C$) in a 12 : 12 hr light/dark cycle. Throughout the course of this study, the rats had access *ad libitum* to water and commercial rat feed (Specialty Feeds, Glen Forrest, Australia).

The animal handling and experimental procedures were carried out as per the Guidelines for Care and Use of Laboratory Animals (ARRIVE guidelines). This study was approved by the UiTM Committee on Animal Research and Ethics (UiTM CARE 163/2017). It was conducted according to the Malaysian Animal Welfare Act 2015 (AWA 2015) as well as the Animal (Amendment) Act 2013 (AA 2013) [20]. The present study was also in compliance with The Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes (MY CODE) that was adopted and adapted (with consent from the National Health and Medical Research Council-Australia) from the Australian Code for the Care and Use of Animals for Scientific Purposes, 7th and 8th Editions ([21] and [22], respectively).

2.2. Study Design. Twenty-four, adult, Sprague-Dawley rats were randomized equally into four groups. Rats in Group 1 (control) were administered 0.1 ml normal saline intraperitoneally, while those in Group 2 (leptin) were given leptin at a dose of $60 \mu g/kg/day$ [15] intraperitoneally. Animals in Group 3 (Profortil® + leptin) were administered both Profortil® at a dose of 50 mg/kg/day via oral gavage and leptin at $60 \mu g/kg/day$ intraperitoneally. Group 4 (Profortil®) rats were given just Profortil® at a dose of 50 mg/kg/day via oral gavage. Leptin was administered once daily for 2 weeks. Profortil® was given for 3 weeks: as a pretreatment for 1 week, followed by 2 weeks concurrently with leptin.

TABLE 1: Composition of Profortil®.

Nutritional information	Per serving (two capsules)
Energy	11.8 kJ/2.8 kcal
L-Carnitine	440 mg
L-Arginine	250 mg
Vitamin E	120 mg
Glutathione	80 mg
Zinc	40 mg
Coenzyme Q10	15 mg
Folic acid (vitamin B9)	800 mcg
Selenium	60 mcg

For intraperitoneal injection, leptin (rat recombinant, BioVision Incorporated, Waltham, USA) in lyophilized powder form was first reconstituted in distilled water to a concentration of 0.1 mg/ml. This leptin solution was diluted in normal saline to the appropriate concentration, and then given in a volume of 0.1 ml, similar to that given to the controls. The leptin dose used, its route of administration (intraperitoneal), and the time of day it was injected (between 9 am and 10 am) were all based on our previous studies [8, 15, 17]. For oral gavage, one capsule of Profortil® (Table 1) was opened and the contents were weighed. According to the weight of the rat, 50 mg/kg Profortil® were mixed with 0.5 ml of normal saline, vortexed, and administered via oral gavage immediately. A fresh solution was prepared for each administration.

The body weight and food intake of the rats were recorded weekly. Once the treatment period concluded, the rats were euthanized via an overdose of sodium pentobarbital by intraperitoneal injection. Laparotomy was performed, following which the testes and epididymides were collected and weighed. Sperm were collected from the rat cauda epididymides for assessment of sperm parameters and Comet assay. The left testis was immersed at once in 10% neutral buffered formalin and used for the TUNEL assay. The right testis was stored at $-80°C$ for assessment of 8-OHdG concentration and antioxidant enzyme activity at a later date.

2.3. Total Sperm Count and Sperm with Abnormal Morphology. The cauda epididymis of each rat was minced in 2 ml of normal saline and the mixture was strained through an $80 \mu m$ nylon mesh. The strained solution was used for the estimation of total sperm count and the fraction of sperm with abnormal morphology using a Makler Counting Chamber (Sefi Medical Instruments Ltd., Haifa, Israel). For this, a drop of the strained solution was placed in the center of the glass slide and covered with a coverslip to allow the drop to spread evenly. The total number of sperm was counted in a strip of 10 squares. This count was repeated two more times and an average was calculated and recorded. For each sample, three drops were analyzed. For calculation of the fraction of sperm with abnormal morphology, morphologically abnormal sperm were observed in the same strip of 10 squares and expressed as a percentage of the total sperm count. Sperm that were considered abnormal included those that were headless, hookless, coiled or

broken tail, acute bending at the cephalocaudal junction, double-headed, double-tailed, and microcephalic [13, 15, 17].

2.4. Assessment of Oxidative Stress

2.4.1. Concentration of 8-OHdG. Testicular tissue for the measurement of 8-OHdG concentration was prepared as follows. Briefly, 30 mg of tissue were removed from each harvested right testis and phosphate buffer solution was added to each sample (1 : 9) in an Eppendorf tube. The suspension was well homogenized and then centrifuged at 5,000x g for 5 min, after which the supernatant was collected for ELISA. The concentration of 8-OHdG in the testicular tissue was measured using the Elabscience 8-OHdG ELISA kit (E-EL-0028, Elabscience Biotechnology, Texas, USA) according to the manufacturer's protocol.

2.4.2. Superoxide Dismutase and Catalase Activity. The preparation of testicular tissue for the measurement of antioxidant enzyme activity was similar to that for 8-OHdG. Superoxide dismutase (SOD) activity in testicular tissue was measured using the OxiSelect SOD Activity assay kit (STA-340, Cell Biolabs Inc., San Diego, USA) following the manufacturer's protocol. Catalase (CAT) activity in the testicular tissue was measured using the Elabscience Catalase Activity assay kit (E-BC-K031-M, Elabscience Biotechnology, Texas, USA) as per the manufacturer's protocol.

2.5. Testicular Tissue DNA Fragmentation. To identify apoptosis, DNA fragmentation in the testicular tissue was assessed using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The left testes, fixed in formalin, were processed and then embedded in paraffin wax, sectioned into 5 μm sections and mounted onto clear microscope slides. These slides were used for the subsequent TUNEL assay procedure, which was performed using the Elabscience TUNEL assay kit (FITC; E-CK-A333, Elabscience Biotechnology, Texas, USA). To calculate the apoptotic index, the number of TUNEL-positive germ cells per seminiferous tubule was divided by the total number of germ cells per seminiferous tubule, and then multiplied by 100. A total of 1,000 cells were counted in each section view.

2.6. Sperm DNA Damage. DNA damage in the spermatozoa was assessed using the Comet assay according to the previously described procedure from our lab [15]. Fully frosted slides were covered with 100 μl of 1% normal melting agarose. Coverslips were carefully placed on the slides, which were then kept at $-4°C$ for 5–10 min. Once the agarose had been set, the coverslips were removed. A sperm sample, containing a concentration of 1×10^5 sperm was pipetted onto the slide, followed by 90 μl of 1% low melting agarose. The slides were then covered again and kept at $-4°C$ for 5–10 min. Subsequently, the covers were removed, and the slides submerged in lysis buffer at 37°C for 24 hr. Next, these slides were washed with deionized water and then placed in an electrophoresis buffer contained in an electrophoresis tank. Electrophoresis was performed for 20 min at 300 mA, 25 V. Following this, slides were washed with a neutralization buffer and then stained with SYBR green-1 (diluted 1 :

10,000) for 30 min in a dark room. The slides were then immediately viewed under a fluorescence microscope, and the captured images were analyzed using Comet assay analysis software (CASPLab version 1.2.3beta2, CaspLab, Wroclaw, Poland). In the resulting Comet structure, the "head" contains the undamaged DNA nucleoid body, while the "tail" represents the trailing damaged DNA streak. Two hundred comets from each sample were analyzed. For each comet, the tail length, percentage of tail DNA (which is directly proportional to the percentage of DNA damage), tail moment, and olive tail moment were scored. The tail length, which is the DNA migration distance from the nuclear core body, represents the extent of DNA damage. The tail DNA percentage is directly proportional to the percentage of DNA damage. The tail moment determines the number of broken or relaxed pieces (tail DNA intensity) along with the smallest detectable size of migrating DNA (comet tail length). The olive tail moment is a product of the tail length and the total tail DNA fraction [23].

2.7. Statistical Analysis. Data analysis was performed using SPSS software version 20.0 (IBM Corp., Armonk, New York, USA). The Shapiro–Wilk test was used to test for the normal distribution of the data. Since all data were normally distributed, parametric tests were used for further analysis, one-way analysis of variance (ANOVA) and subsequently Tukey's post-hoc analysis. Data are expressed as mean $\pm$ SEM. A $p < 0.05$ was considered significant.

3. Results

3.1. Effects of Profortil® and Leptin on Sperm Parameters. Total sperm count was significantly higher in the Profortil® + leptin ($p < 0.05$) and Profortil®-only ($p < 0.01$) treated groups when compared with that in the leptin-only treated group (Figure 1(a)). Total sperm count was significantly ($p < 0.05$) lower in the leptin-only treated group when compared to that in the control. There were no significant differences observed in sperm count between the control, Profortil® + leptin, and Profortil®-only treated groups.

Although there were no statistically significant differences observed in the fraction of sperm with abnormal morphology between the leptin-only and Profortil® + leptin-treated groups, the fraction was nevertheless lower in the latter group (Figure 1(b)). It was also significantly ($p < 0.05$) lower in the Profortil®-only treated group when compared with that in the leptin-only treated group. The fraction of sperm with abnormal morphology was significantly ($p < 0.05$) higher in the leptin-only treated group when compared with that in the control group. There were no significant differences observed between the control and Profortil®-only groups.

3.2. Effects of Profortil® and Leptin on 8-OHdG, SOD Activity, and CAT Activity. In the Profortil® + leptin-treated group, testicular 8-OHdG concentration was significantly ($p < 0.05$) reduced when compared with that in the leptin-only treated group (Figure 2). Testicular 8-OHdG was significantly ($p < 0.05$) higher in the leptin-only treated group compared to the control group. No significant difference was observed
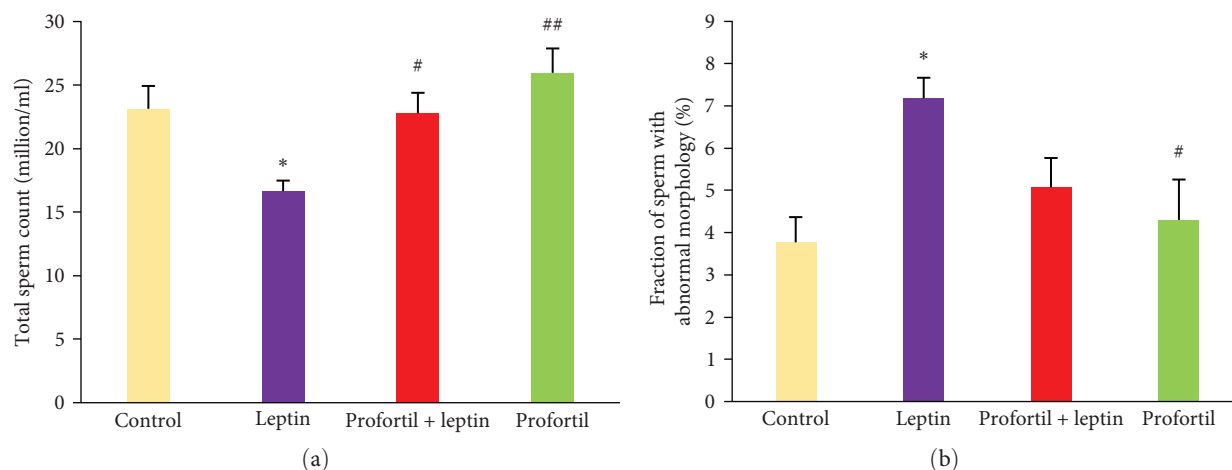
(a)

(b)

FIGURE 1: Effects of Profortil® and leptin on: (a) total sperm count and (b) fraction of sperm with abnormal morphology; $n = 6$ for each group. $^*p < 0.05$ compared to control group. $^\#p < 0.05$; $^{\#\#}p < 0.01$ compared to leptin-only treated group.
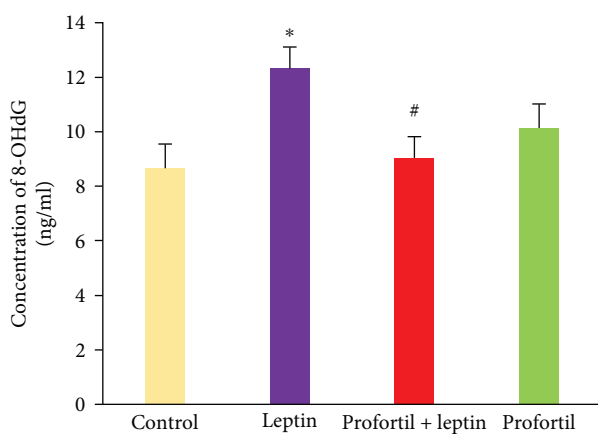

FIGURE 2: Effects of Profortil® and leptin on the concentration of 8-OHdG in testes; $n = 6$ for each group. $^*p < 0.05$ compared to control. $^\#p < 0.05$ compared to leptin.

between the control, Profortil® + leptin, and Profortil®-only treated groups.

Regarding SOD activity in the testicular tissues, there was no significant difference observed between the four groups (Figure 3(a)). CAT activity was significantly ($p < 0.05$) lower in the leptin-only treated group than that in the control group (Figure 3(b)). It was, however, not significantly different between the Profortil® + leptin, leptin-only, and Profortil®-only treated groups. There were no significant differences observed between the control, Profortil® + leptin, and Profortil®-only groups.

3.3. Effects of Profortil® and Leptin on Testicular DNA Fragmentation. Testicular DNA fragmentation was significantly higher in the leptin-only and Profortil® + leptin treated groups compared with the control ($p < 0.001$, respectively) and Profortil®-only treated groups ($p < 0.001$; Figure 4). There was no significant difference observed between the leptin and Profortil® + leptin-treated groups nor the control and Profortil®-only groups.

3.4. Effects of Profortil® and Leptin on Sperm DNA Damage. No significant difference was noted in the percentage of DNA in the Comet tail between all the groups, although it was slightly higher in the leptin, Profortil® + leptin, and Profortil®-only treated groups (Figure 5(a)). Comet tail length was significantly longer in the leptin and Profortil® + leptin groups ($p < 0.05$, respectively) compared with that in the control group (Figure 5(b)). There was no significant difference seen between the control and Profortil®-only groups.

No significant difference was observed in the tail moment score between all the groups (Figure 5(c)). Olive tail moment score was not significantly different between the leptin-only, Profortil® + leptin, and Profortil®-only treated groups (Figure 5(d)). However, it was significantly ($p < 0.05$) higher in the leptin-only treated group compared to that in the control group. There were no significant differences observed between the control, Profortil® + leptin, and Profortil® groups (Figure 6(a)–6(d)).

3.5. Effects of Profortil® and Leptin on Food Intake, Body Weight, and Organ Weight. No significant difference was observed in food intake between all groups when measured at the start and the end of the treatment duration (Table 2). There was also no significant difference in mean body weight between the groups either at the beginning or at the end of treatment (Table 3) as well as the weight of the testes and cauda epididymides between the four groups at the end of the treatment duration (Table 4).

4. Discussion

To the best of our knowledge, this is the first study investigating the effects of Profortil® on leptin-induced adverse effects on rat spermatozoa. In this study, Profortil® supplementation to leptin-treated rats was able to prevent the leptin-induced reduction in total sperm count and a rise in the concentration of 8-OHdG in the testicular tissue. It, however, did not have significant effects on leptin-induced changes in the fraction of sperm with abnormal morphology,
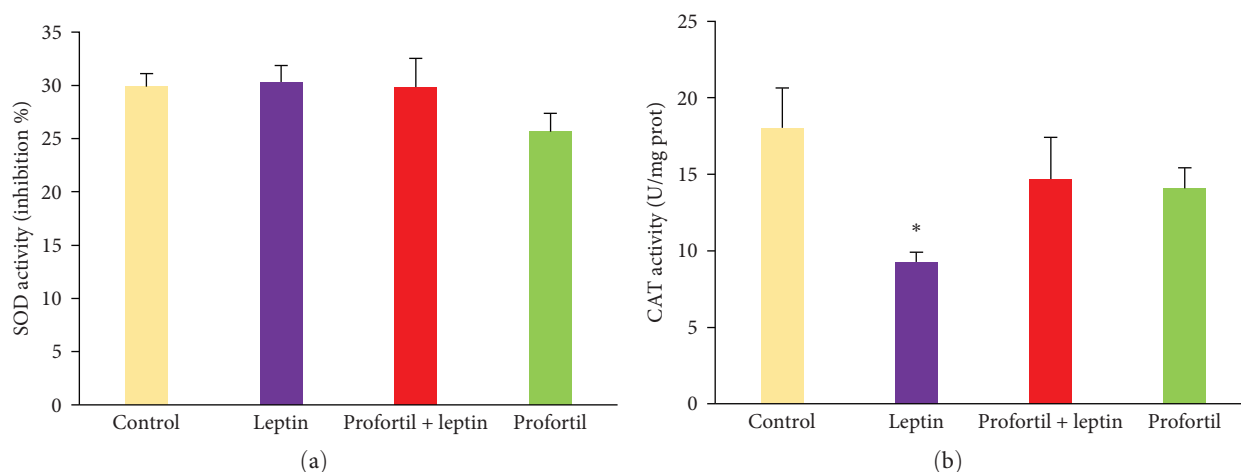
FIGURE 3: Effects of Profortil® and leptin on antioxidant enzyme activities in the testis: (a) superoxide dismutase (SOD) activity and (b) catalase (CAT) activity; $n = 6$ for each group. $^*p < 0.05$ compared to control.
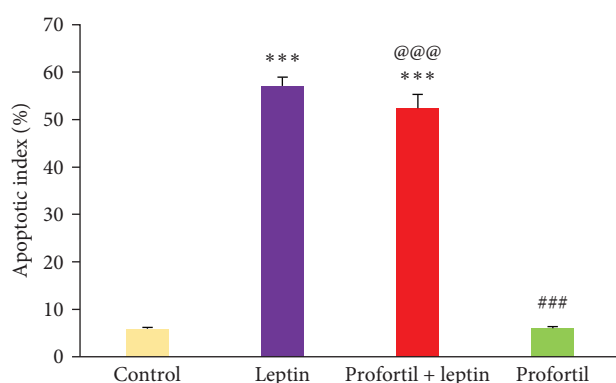

FIGURE 4: Effects of Profortil® and leptin on testicular DNA fragmentation. The apoptotic index was calculated using the TUNEL assay; $n = 6$ for each group. $^{***}p < 0.001$ compared to control. $^{###}p < 0.001$ compared to leptin. $^{@@@}p < 0.001$ compared to Profortil®.

SOD and CAT activities, testicular DNA fragmentation, and sperm DNA damage. The precise reason for these selective preventive effects of Profortil® is uncertain.

In this study, Profortil® treatment for 3 weeks was only able to prevent some of the effects of leptin on sperm and testes. Profortil® seems to have prevented the decrease in sperm count in leptin-treated rats, as evident from the significantly higher sperm count in the leptin + Profortil®-treated rats when compared with that in leptin-only treated rats (Figure 1(a)). Although there was no significant difference observed in the fraction of sperm with abnormal morphology, the percentage was, nevertheless, slightly lower than that in the leptin-only treated rats (Figure 1(b)). For it to have a significant effect on sperm morphology, it is possible that a longer treatment duration or a higher Profortil® dose may be required.

The duration from the initiation of stem cell division to the formation of mature spermatozoa in rats is 52 days [24]. As has been suggested by Almabhouh et al. [8], the effects of leptin may include effects on some of the upstream processes in spermatogenesis. Thus, a longer duration of treatment

with Profortil®, for example, 8 weeks or longer, may have likely resulted in a more significant reduction in the fraction of sperm with abnormal morphology, as previously reported with melatonin [8]. Besides that, it has also been reported that the adverse effects of 6 weeks of leptin treatment take about 6–8 weeks to reverse, suggesting that the action of leptin also includes upstream stages of spermatogenesis [8].

The effects of Profortil® treatment on basic sperm parameters have previously been investigated in several human studies, with mixed results. For example, treatment with two capsules of Profortil® daily for 3 months in subfertile males was shown to have increased ejaculatory volume, total and progressive motility, and sperm density, decreased abnormal sperm morphology, and improved pregnancy outcomes [2]. Another study in sub-/infertile males given the same treatment regime also observed an increase in sperm density and motility, but not in sperm morphology [25]. However, when males with idiopathic infertility were treated with the same dose of Profortil® for a shorter period of 6 weeks, no significant changes were found in sperm count and motility compared to the control group [4].

Adverse effects of exogenous leptin administration on spermatozoa and testicular tissue have already been shown by several animal studies [8, 13–15, 17]. It has also already been reported that these effects involve the PI3K pathway [17]. The biomarker 8-OHdG is a commonly used marker for assessing oxidative stress-induced DNA damage. Its high levels, as evidenced in the testicular tissue (Figure 2), show that oxidative stress is induced by leptin, and this consequently led to testicular DNA fragmentation and sperm DNA damage (Figures 4 and 5). In turn, this could result in a significant decrease in the total sperm count and an increase in the fraction of sperm with abnormal morphology. The harmful effects of oxidative stress on sperm count and morphology are well established [26].

In this study, treatment with Profortil® in leptin-induced rats caused a significant decrease in 8-OHdG concentration (Figure 2). However, the apoptotic index of the testicular tissues did not differ significantly between leptin and leptin
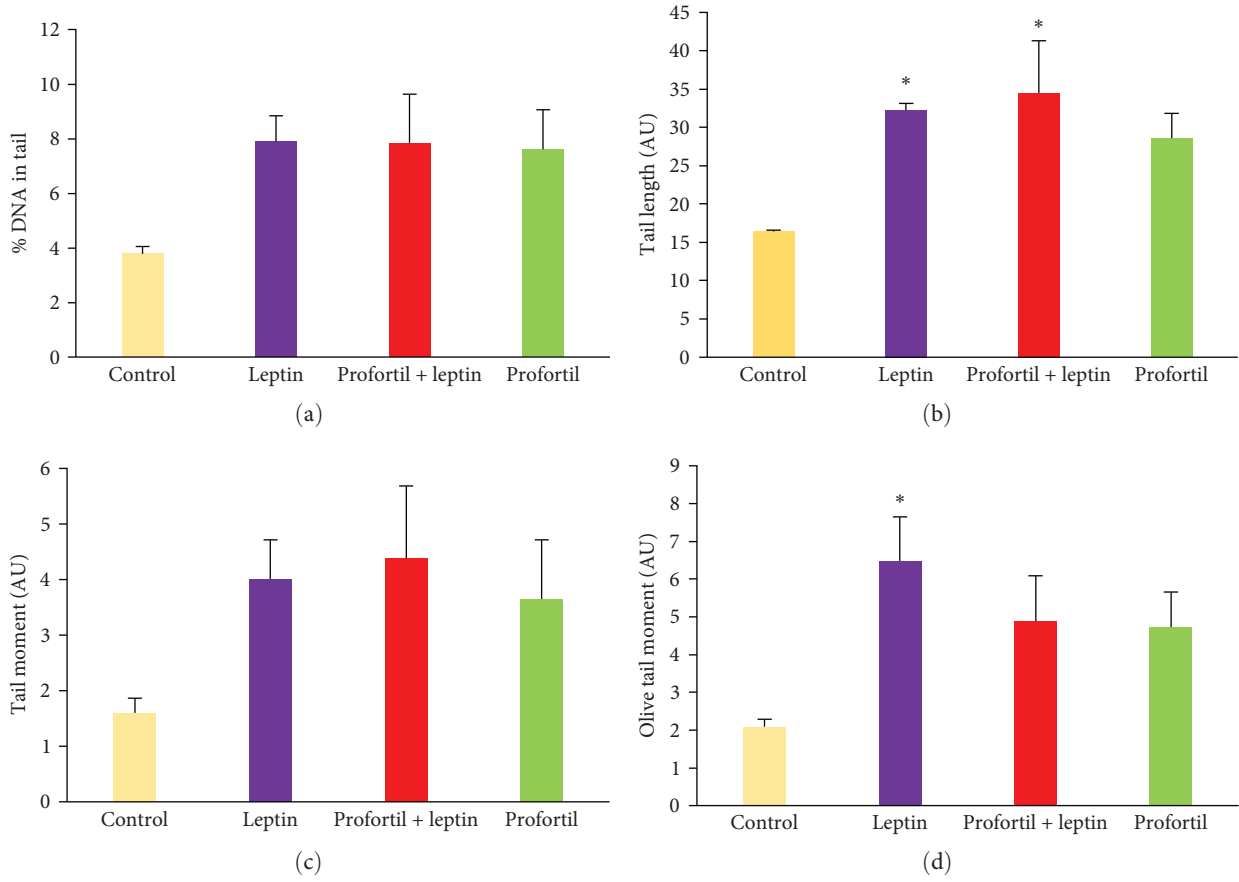
FIGURE 5: Effects of Profortil® and leptin on sperm DNA damage assessed by the Comet assay: (a) percentage DNA in tail of comet, (b) tail length score, (c) tail moment score, and (d) olive tail moment; $n = 6$ for each group. $*p < 0.05$ compared to control.
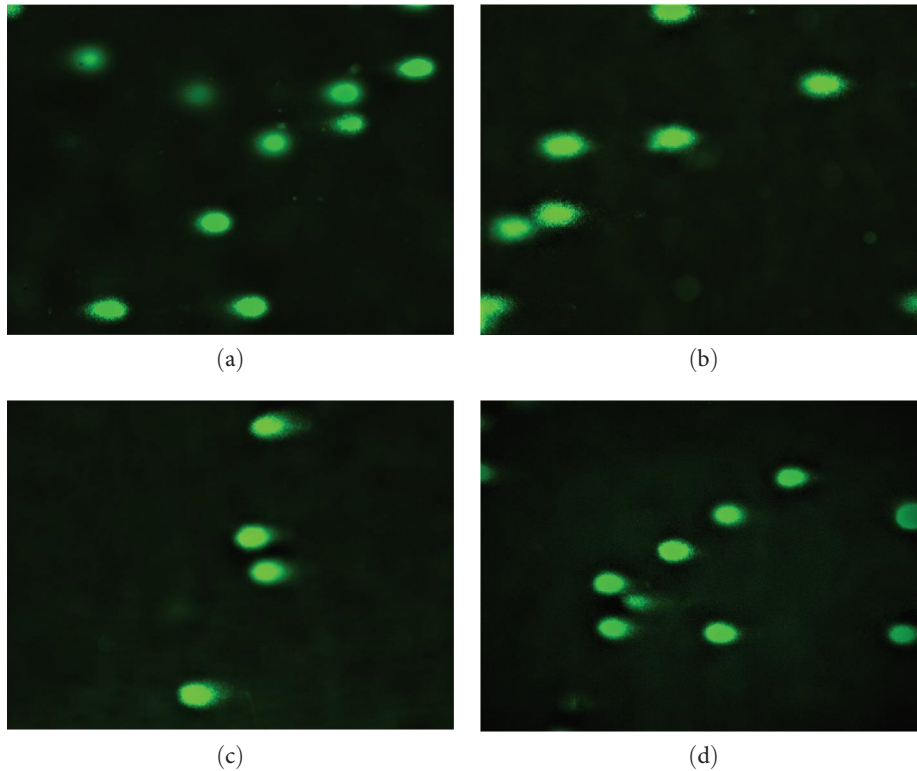

FIGURE 6: Effects of Profortil® and leptin on sperm DNA damage. Photomicrograph showing Comet assay of sperm: (a) control, (b) leptin, (c) Profortil® + Leptin, and (d) Profortil®.

TABLE 2: Effects of Profortil® and leptin on food intake (g) of the rats at the beginning and end of treatment.

Groups ($n = 6$ for each group)	Beginning of treatment (g)	End of treatment (g)
Control	21.33 ± 0.80	21.15 ± 0.79
Leptin	20.67 ± 0.94	19.88 ± 0.67
Profortil® + Leptin	19.14 ± 0.89	19.86 ± 2.14
Profortil®	20.17 ± 1.04	20.00 ± 1.51

TABLE 3: Effects of Profortil® and leptin on body weight (g) of the rats at the beginning and end of treatment.

Groups ($n = 6$ for each group)	Beginning of treatment (g)	End of treatment (g)
Control	346.33 ± 20.04	377.67 ± 20.81
Leptin	326.50 ± 10.13	359.13 ± 11.60
Profortil® + Leptin	383.71 ± 10.99	401.14 ± 19.14
Profortil®	392.50 ± 12.97	404.00 ± 16.70

TABLE 4: Effects of Profortil® and leptin on the weight (g) of testes and cauda of epididymides in the rats after treatment.

Groups ($n = 6$ for each group)	Right testes (g)	Left testes (g)	Right cauda (g)	Left cauda (g)
Control	1.53 ± 0.08	1.53 ± 0.07	0.24 ± 0.01	0.23 ± 0.01
Leptin	1.47 ± 0.02	1.46 ± 0.02	0.24 ± 0.01	0.23 ± 0.01
Profortil® + Leptin	1.61 ± 0.05	1.60 ± 0.05	0.26 ± 0.01	0.27 ± 0.01
Profortil®	1.60 ± 0.05	1.60 ± 0.06	0.25 ± 0.02	0.26 ± 0.01

+ Profortil®-treated groups (Figure 4). Sperm DNA damage in the leptin-treated rats was also not significantly different than in the Profortil® treated rats (Figure 5). The precise significance of this is unclear but it might suggest that the level of its antioxidant activity might have been insufficient to lower sperm DNA damage and the testicular DNA fragmentation adequately. In a study similar to ours, melatonin administration for 42 days in leptin-treated Sprague-Dawley rats showed a significant improvement in sperm parameters, a decrease in sperm DNA damage, and a decrease in testicular DNA fragmentation [8]. When compared to Profortil®, melatonin appears to be more effective in preventing these leptin-induced adverse effects. However, that cannot be concluded with certainty as melatonin was given for a much longer duration than was Profortil® (6 weeks vs. 2 weeks in our study), and may have contributed to its effectiveness in preventing leptin-induced adverse effects on the sperm parameters. Perhaps a higher dose of Profortil®, and/or a longer duration of treatment of more than 3 weeks might have given more significant benefits.

Treatment with leptin in the Sprague-Dawley rats resulted in a significant decrease in CAT activity, but had no effect on SOD activity (Figures 3(a) and 3(b)). Profortil® treatment in leptin-induced rats did not affect the SOD activity. Additionally, no significant difference was observed in the CAT activity between the leptin-only and Profortil® + leptin-treated rats; however, the activity of CAT was slightly higher (Figure 3(b)). In the study by Almabhouh et al. [8], leptin treatment also resulted in a downregulation in CAT but had upregulated SOD genes in the testes of the SD rats.

The administration of melatonin in those rats also caused an upregulation in SOD genes. The reason for the slight differences in the CAT and SOD activities is unclear.

5. Conclusion

In conclusion, Profortil® administration, at a dose of 50 mg/kg/day for 3 weeks to leptin-treated Sprague-Dawley rats, was able to decrease the markers for oxidative stress as shown by the decrease in the concentration of testicular 8-OHdG in the Profortil® + leptin group but was not able to prevent all the affected parameters as was the case with melatonin. Nevertheless, Profortil® may have the potential to prevent leptin-induced adverse effects on sperm parameters. Further studies using a longer duration of administration or a higher dose of Profortil® may provide more conclusive results and a better comparison with the effects of melatonin.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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