Melatonin ameliorates the adverse effects of leptin on sperm

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This study examined the effects of melatonin on leptin-induced changes in sperm parameters in adult rats. Five groups of Sprague-Dawley rats were treated with either leptin or leptin and melatonin or melatonin for 6 weeks. Leptin was given daily via the intraperitoneal route (60 µg kg⁻¹ body weight) and melatonin was given in drinking water (10 mg kg⁻¹ or 20 mg kg⁻¹ body weight per day). Upon completion, sperm count, sperm morphology, 8-hydroxy-2-deoxyguanosine, Comet assay, TUNEL assay, gene expression profiles of antioxidant enzymes, respiratory chain reaction enzymes, DNA damage, and apoptosis genes were estimated. Data were analyzed using ANOVA. Sperm count was significantly lower whereas the fraction of sperm with abnormal morphology, the level of 8-hydroxy-2-deoxyguanosine, and sperm DNA fragmentation were significantly higher in rats treated with leptin only. Microarray analysis revealed significant upregulation of apoptosis-inducing factor, histone acetyl transferase, respiratory chain reaction enzyme, cell necrosis and DNA repair genes, and downregulation of antioxidant enzyme genes in leptin-treated rats. Real-time polymerase chain reaction showed significant decreases in glutathione peroxidase 1 expression with increases in the expression of apoptosis-inducing factor and histone acetyl transferase in leptin-treated rats. There was no change in the gene expression of caspase-3 (CASP-3). In conclusion, the adverse effects of leptin on sperm can be prevented by concurrent melatonin administration.

Keywords: 8-hydroxy-2-deoxyguanosine; DNA fragmentation; leptin; melatonin; sperm

INTRODUCTION

Leptin, a 16-kDa protein that is produced and secreted mainly by the adipose tissue, is a hormone that has been shown to have roles in diverse physiological processes including regulation of body weight and food intake, immune function, hematopoiesis, inflammation, sexual maturation, and normal reproduction.1-4 However, a number of recent reports have indicated some adverse effects of leptin on sperm count and morphology. Exogenous leptin administration to normal rats for 6 weeks was found to decrease sperm concentration while increasing the fraction of sperm with abnormal morphology.5-8 Although the precise mechanism for this remains unclear, leptin-induced oxidative stress has been implicated, as leptin administration has been shown to increase free radical production.9 Reactive oxygen species and their metabolites cause damage to the membrane lipids, DNA, and cellular proteins, and oxidative stress is now hypothesized as a possible cause of male infertility.10-13 While increased free radical production might be responsible for the leptin-induced decreases in sperm count and abnormal sperm morphology, it, however, remains unknown if concurrent antioxidant supplementation to leptin treated rats would prevent the adverse effects of leptin on some of these sperm parameters. Melatonin interacts with various reactive oxygen and reactive nitrogen species and also upregulates antioxidant enzymes and downregulates pro-oxidant enzymes. Therefore, diverse beneficial effects of melatonin have been claimed to protect against various degenerative conditions caused by oxidative stress. This study examined the effects of concurrent leptin treatment and melatonin supplementation on sperm count, sperm morphology, apoptosis, and DNA damage in normal adult rats.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats, aged 12 weeks, were obtained from the Laboratory Animal Care Unit (LACU), Universiti Teknologi MARA. Rats were housed in standard rat cages with commercial wood chip bedding at room temperature and with a 12/12 light/dark cycle. Rats had access ad libitum to commercial rat feed (Specialty Feeds Pty Ltd., Perth, Australia) and tap water throughout the experimental period. The experimental protocol used in this study was approved by the Animal Care and Use Committee (ACUC), Universiti Teknologi MARA. Rats were randomized into five groups consisting of control, leptin, leptin-melatonin-10 (LM10), leptin-melatonin-20 (LM20), and melatonin-10 (M10) treated groups with 6 rats per group. All leptin-treated groups received intraperitoneal injections (i.p.) of leptin once daily for 42 days (60 µg kg⁻¹ body weight; recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombinant rat leptin, recombi
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Comet assay was performed on sperm to assess its DNA integrity. Sperm Comet assay was used to estimate 8-OHdG. EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) was used to prepare amplified cDNA with reagents provided in the manufacturer's instructions (BioVision Inc., Milpitas, California, USA). The cDNA was purified with QIAGEN's MinElute reaction and concentration were ascertained. About 200 ng of total RNA was used to prepare amplified cDNA with reagents provided in the manufacture's protocol, followed by treatment with DNase (Thermo Scientific, Foster, CA, USA). The RNA quality and concentration were ascertained. About 200 ng of total RNA was used to prepare amplified cDNA with reagents provided in the Appliance WT-Amp plus ST System Kit (Nugen Technology, San Carlos, CA, USA). The CDNA was purified with QiAGEN's MinElute reaction Cleanup Kit (QiAGEN, Hilden, Germany) and its concentration and purity were determined. The Encore Biotin Module (Part No. 4200) was used to label the CDNA and then hybridized using GeneChip Hybridization kit (Affymetrix Rat GeneChip St 2) for 18 h in the GeneChip Hybridization Oven 640 (Affymetrix, Santa Clara, CA, USA). Immediately following hybridization, the array was washed and stained with streptavidin phycoerythrin conjugate on the
GeneChip Fluidic Station 450 (Affymetrix, Santa Clara, CA, USA), followed by scanning on a GeneChip Scanner. Data from microarray were displayed as CEL, DAT, and JPG files. These output data from microarray were analyzed as gene-level differential expression by Affymetrix software (Expression Console-1.3-1-64 bit and Transcriptome Analysis Console-2-0-64 bit). Gene expressions in leptin- and melatonin-treated rats that were either 2 fold greater or 2-fold lesser than those in the control were considered significant ($P < 0.05$). Upregulated and downregulated genes were then grouped according to their functions using software (TIBCO Spotfire® software Version 1.0.0, Palo Alto, CA, USA) Server: https://spotfire.cloud.tibco.com/.

**Real-time PCR**

Gene expressions of caspase-3 (CASP-3), apoptosis-inducing factor (AIF), glutathione peroxidase 1 (GPX1), and histone acetyltransferases (HAT) that were found altered in the cDNA microarray analysis were further confirmed by real-time polymerase chain reaction (PCR). Briefly, total RNA was extracted from testicular tissue with the aid of innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Then, 2 µl RNA (30 ng) was used in a 20 µl cDNA reaction using 5x iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The cDNA was further PCR-amplified by specific primers in 10 µl PCR mixture (SYBR Green Supermix and 25 µmol l$^{-1}$ forward and reverse primers template) with q$^\circ$ 5 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The paired primer sets included forward CASP-3 (5'-TGGAAGGTGATTTATGGGACA3'), CASP-3 reverse (5'-TCCCATATAATTGACCCCTCTCATCA3'), forward AIF (5'-CCACACGGAGAAGCTTGTTGTATC3') and reverse AIF (5'-GATCATGGACTGTGGTCATGA3'), reverse GPX1 (5'-GGCATGGACTGTGGTCATGA3') and TATA box binding protein (TBP) forward (5'-CAACTTCCATCAAGTTGCA3') and reverse (5'-GGATGATGTTTTTACCA3'), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) forward (5'-CAACTTCCATCAAGTTGCA3') and reverse (5'-GGATGATGTTTTTACCA3'), and TATA box binding protein (TBP) forward (5'-CAACTTCCATCAAGTTGCA3') and reverse (5'-GGATGATGTTTTTACCA3'). Normalization of gene expression was done with GAPDH and TBP housekeeping genes. The q$^\circ$ 5 Real-Time PCR software (Bio-Rad, Hercules, CA, USA) was used to calculate the gene expression for all samples.

**Statistical analysis**

ANOVA with Tukey's post hoc test contained in SPSS version 21 (SPSS Inc., Chicago, Illinois, USA) was used to analyze the data. The data are expressed as mean ± standard deviation. A significant difference was accepted when $P < 0.05$.

**RESULTS**

Body weight increased in all rats over the 6-week study period (Table 1). However, no differences were evident in body weight between leptin, LM10, LM20, melatonin-only treated groups, and that of the control group.

Total sperm count was lower in leptin-only and LM10-treated rats than that in the control ($P < 0.001$ and $P < 0.01$, respectively, Figure 1a). No difference was evident in sperm count between control and LM20- and M10-treated rats.

The fraction of sperm with abnormal morphology was higher in leptin only-treated rats compared to that in the control ($P < 0.001$; Figure 1b).

No differences were evident in the fraction of sperm with abnormal morphology between M10, LM10, LM20, and that of saline-treated control group. The major morphologically abnormal sperm noticeable was the headless and coiled tail types.

TUNEL assay revealed evidence of higher DNA fragmentation and apoptosis in the seminiferous tubules of leptin-treated rats than that in the control rats (Figure 2a). In addition, the AI in leptin-treated rats was higher than that in the control rats (Figure 2f). There was no difference in the AI between controls and rats given melatonin at a dose of 20 mg kg$^{-1}$ body weight.

No difference was evident in the level of sperm 8-OHdG between control and LM20- and M10-treated rats. However, 8-OHdG level was higher in leptin-only and LM10-treated rats than that in controls ($P < 0.001$ and $P < 0.05$, respectively, Figure 3).

Tail length, tail moment, olive tail moment, and % tail DNA were higher in leptin-only, LM10-, and LM20-treated rats than that in the controls (Table 2). These four parameters were higher in leptin- and LM10-treated rats than that in the LM20-treated rats.

Microarray analysis revealed 1893 and 1119 genes that were upregulated in leptin-treated rats and LM20-treated rats, respectively. A total of 3800 and 898 genes were downregulated in leptin- and LM20-treated rats, respectively, when compared with the expressions in the controls. Of these, some of the genes regulating apoptosis, DNA damage, DNA repair, cell necrosis, antioxidant enzymes, and respiratory chain reaction enzymes are shown in Table 3.

**Table 1: Body weight in control and leptin-treated rats**

| Groups       | Body weight (g) ± s.d. | Day 0                | Day 42                |
|--------------|------------------------|----------------------|-----------------------|
| Control      | 345.83±11.13           | 412.67±15.81***      |
| Leptin       | 344.33±12.26           | 410.00±12.60***      |
| Leptin-melan10 | 345.52±5.43           | 410.00±15.59***      |
| Leptin-melan20 | 339.00±5.85           | 403.67±2.19***       |
| Melatonin    | 342.00±3.98            | 413.33±9.73***       |

**Table 2: Sperm with ssDNA break in rat of experimental groups shown by Comet assay (mean±s.d.)**

| Groups       | Tail length (arbitrary unit) | Tail moment (arbitrary unit) | Olive tail moment (arbitrary unit) | % tail DNA (arbitrary unit) |
|--------------|-----------------------------|-----------------------------|-----------------------------------|-----------------------------|
| Control      | 19.25±4.93                  | 2.09±0.88                   | 2.25±0.37                         | 0.88±0.09                   |
| Leptin       | 39.87±2.41**                | 7.19±1.00**                 | 6.56±0.81**                       | 1.61±0.25**                 |
| LM-10        | 34.06±3.53**                | 6.08±0.26**                 | 5.36±0.84**                       | 1.24±0.18**                 |
| LM-20        | 22.75±2.62                  | 3.09±0.59                   | 3.09±0.13                         | 1.07±0.05                   |
| M-10         | 20.89±1.69                  | 3.04±0.68                   | 2.84±0.33                         | 0.95±0.01                   |

**Figure 1:** Sperm count (a) and sperm morphology (b) in leptin- and melatonin-treated rats compared to control. **P<0.01; ***P<0.001.
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The major findings of this study include: (a) lower total sperm count in leptin-treated rats than in saline-treated controls, (b) higher fraction of sperm with abnormal morphology in leptin-treated rats, (c) higher apoptotic activity and DNA fragmentation in leptin-treated rats, (d) higher levels of 8-OHdG in leptin-treated rats, (e) differential expression of a number of genes following leptin and melatonin treatment, and (f) no significant differences in sperm count, abnormal sperm morphology, apoptotic activity, DNA fragmentation, and 8-OHdG levels between controls and leptin + melatonin-treated rats.

The precise mechanism for the lower total sperm count and higher fraction of sperm with abnormal morphology following leptin treatment is still unclear. Decreases in sperm count and increased fraction of sperm with abnormal morphology following leptin treatment have been reported recently.8,9 Increased sperm 8-OHdG, a marker of DNA damage due to oxidative stress in rats, and increased intracellular levels of reactive oxygen species (ROS) following leptin treatment have been reported recently.7,8 The main sources of ROS are seminiferous tubules and spermatozoa, and low levels of ROS are necessary for normal sperm functions including capacitation, hyperactivation, and acrosome reaction. However, when produced in large amounts, it is detrimental to sperm and germinal cells. Leptin has also been shown to increase superoxide anion production (O2-) in vascular smooth muscle cells in culture and in aortic endothelial cells via protein kinase-A-mediated fatty acid oxidation.10 It also causes peroxynitrite-mediated oxidative stress in steatohepatic lesions.20 Leptin-induced ROS production in mouse hepatocytes is inhibited by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, MAP kinase/ERK kinase 1 (MEK1), and Janus kinase 2 (JAK2) inhibitors, suggesting the possible involvement of these pathways in leptin-induced ROS production.21

The expression of HAT and histone deacetylase (HDAC) was significantly higher in leptin-treated rats than that in control and LM20-treated rats. Leptin has been shown to increase the involvement of histone acetyl transferases in growth-stimulating activity of breast cancer cells.22 Histone acetylation plays an important role in spermatogenesis.23 Histone acetylation of lysine residues, clustered at the amino-terminal end of core histones, is regulated by HAT that facilitate acetylation, and HDAC that decrease acetylation.24,25 A recent study demonstrated a significant positive relationship between HAT activity and sperm DNA fragmentation index.26 When histones are acetylated, their affinity to DNA is decreased by the loss of the positive charge. This then loosens the DNA chromatin structure or makes it less compact for active transcription.25 However, if histones bound to DNA are hyperacetylated, the DNA chromatin becomes further less compact and becomes more susceptible to fragmentation.

The impact of leptin on DNA damage possibly occurs through leptin-induced increase in production of ROS. In this regard, microarray analysis results revealed a significant upregulation of the electron transfer chain enzymes in mitochondria and downregulation of the expression of antioxidant enzymes, catalase (CAT), glutathione peroxidase 1 (GPX1), peroxiredoxin 1 (Prdx1), and glutathione S-transferase pi 1 (Gstpi1) following leptin treatment. This might result in increased production of ROS and oxidative stress, as has been reported recently following leptin treatment.8 The upregulation of HAT gene at the same time might make the DNA less compact and more susceptible to ROS attack, and thereby leading to an increase in DNA fragmentation.

Microarray analysis of the DNA damage genes also revealed an upregulation in the expression of ataxia telangiectasia mutated (ATM), ATM and DNA repair protein rad3-related (ATR), and TP53 and p21 genes in leptin-treated rats. It has been reported that the early molecules that respond to DNA damage consist of ATM and ATR genes.26 The ATM and ATR proteins belong to the phosphatidylinositol 3-kinase-like (PIKK) family of serine/threonine protein kinases and these phosphorylate the DNA damage mediator protein TP53. The target of the transcription factor TP53 is p21, which, in turn, inhibits cyclin-dependent kinase 1 (CDK1) activity, causing arrest of the cell cycle. When the cell cycle arrests, the DNA repair machinery becomes effective.27 If the DNA repair is successful, the cell cycle arrest is lifted, but if the repair is unsuccessful, then programmed cell death

DISCUSSION

Figure 2: Results of TUNEL assay in control (a), leptin-treated (b), leptin-melatonin-10 (LM10) treated (c), leptin-melatonin-20 (LM20) treated (d), melatonin-10 (M10) treated (e) rats, and apoptosis index (f). **P < 0.01; ***P < 0.001; leptin, LM10, LM20, and M20 versus control.

Figure 3: Sperm 8-hydroxy-2-deoxyguanosine (8-OHdG) levels in control, leptin, leptin-melatonin-10 (LM10), leptin-melatonin-20 (LM20), and melatonin-10 (M10) rats. *P < 0.05; ***P < 0.001.
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follows. DNA repair genes, polymerase beta (POLB), ligase III, (Lig3), X-ray repair complementing defective repair (Xrccl), and poly (A) polymerase alpha (Papola) were significantly upregulated following leptin treatment. The increased cell death, as evident from the TUNEL assay, despite the increased expression of DNA repair genes, suggests that the repair was inadequate to sustain the cell cycle in leptin-treated rats. TUNEL assay and Comet assay revealed higher apoptotic activity and DNA fragmentation in rats treated with leptin alone than that in the control and melatonin-treated rats, particularly at a dose of 20 mg kg⁻¹ body weight.

To understand the mechanism of leptin-induced apoptosis, the expression of caspase-dependent and caspase-independent cell death-related genes in the testis was determined. Of the genes involved in caspase-dependent apoptosis, caspase-3, caspase-8, and caspase-9 were

| Table 3: Detailed gene profile analysis by microarray analysis |
|---|
| **Gene symbol** | **Gene description** | **Leptin versus control** | **Leptin-melatonin-20 versus control** |
| **Antioxidant enzymes** | | **Fold change** | **Status** | **Fold change** | **Status** |
| Cat | Catalase | −1.2 | Downregulated | - | Unchanged |
| Prdx1 | Peroxiredoxin 1 | −1.62 | Downregulated | - | Unchanged |
| Gpx7 | Glutathione peroxidase 7 | −1.27 | Downregulated | - | Unchanged |
| Gpx1 | Glutathione peroxidase 1 | −1.28 | Downregulated | - | Unchanged |
| Gsp1 | Glutathione S-transferase pi 1 | −1.3 | Downregulated | - | Unchanged |
| Gstt4 | Glutathione S-transferase, theta 4 | 10.5 | Upregulated | - | Unchanged |
| Sod1 | Superoxide dismutase 1, soluble | 4.37 | Upregulated | 1.97 | Upregulated |
| **Respiratory chain reaction enzymes** | | | | |
| Ndufa3 | NADH dehydrogenase (ubiquinone) complex I, assembly factor 3 | 3.68 | Upregulated | - | Unchanged |
| Sdh | Succinate dehydrogenase complex, subunit B, iron sulfur (lp) | 1.91 | Upregulated | - | Unchanged |
| Uqcrfs1 | Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 | 1.87 | Upregulated | - | Unchanged |
| Cox3 | Cytochrome C oxidase assembly factor 3 | 4.42 | Upregulated | - | Unchanged |
| **DNA damage** | | | | |
| ATM | Ataxia telangiectasia mutated | 1.12 | Upregulated | - | Unchanged |
| ATR | ATM- and rad3-related | 2.22 | Upregulated | - | Unchanged |
| TP53 | Tumor protein p53 | 1.19 | Upregulated | - | Unchanged |
| p21 | Protein p21 | 2.98 | Upregulated | 1.96 | Upregulated |
| Cdk1 | Cyclin-dependent kinase 1 | - | Unchanged | - | Unchanged |
| **DNA repair** | | | | |
| POLB | Polymerase (DNA directed), beta | 4.08 | Upregulated | 2.14 | Upregulated |
| Lig3 | Ligase III, DNA, ATP-dependent | 4.98 | Upregulated | - | Unchanged |
| Xrccl | X-ray repair complementing defective repair in Chinese hamster cells 1 | 1.33 | Upregulated | - | Unchanged |
| Papola | Poly (A) polymerase alpha | 3.54 | Upregulated | - | Unchanged |
| Pcna | Proliferating cell nuclear antigen | 4.55 | Upregulated | - | Unchanged |
| XPC | Xeroderma pigmentosum, complementation group C | 1.34 | Upregulated | - | Unchanged |
| **Cell necrosis** | | | | |
| PARP1 | Poly(ADP-ribose) polymerase 1 | 2.16 | Upregulated | - | Unchanged |
| RIPK1 | Receptor (TNFRSF)-interacting serine-threonine kinase 1 | 1.14 | Upregulated | - | Unchanged |
| TNF | Tumor necrosis factor, alpha | 8.55 | Upregulated | - | Unchanged |
| Jnk | c-Jun N-terminal kinase | 1.99 | Upregulated | - | Unchanged |
| TRAF2 | TNF receptor associated factor 2 | 1.66 | Upregulated | - | Unchanged |
| **Apoptosis** | | | | |
| Casp3 | Caspase-3 | - | Unchanged | - | Unchanged |
| Casp8 | Caspase-8 | - | Unchanged | - | Unchanged |
| Casp9 | Caspase-9 | - | Unchanged | - | Unchanged |
| Bcl2 | Bcl2-like-1 | −2.22 | Downregulated | −2.87 | Downregulated |
| AIF | Apoptosis-inducing factor | 1.44 | Upregulated | - | Unchanged |
| Ctsd | Cathepsin D | 1.49 | Upregulated | - | Unchanged |
| Htra2 | High temperature requirement protein A2 | 1.11 | Upregulated | - | Unchanged |
| **Genes involved in spermatogenesis** | | | | |
| HAT | Histone acetyltransferase | 2.17 | Upregulated | - | Unchanged |
| HDAC1 | Histone deacetylase 1 | 4.3 | Upregulated | - | Unchanged |
| HDAC2 | Histone deacetylase 2 | 4.07 | Upregulated | - | Unchanged |

Rad3: DNA repair protein rad3; ATP: adenosine triphosphate; ADP: adenosine diphosphate; TNFRSF: tumor necrosis factor receptor superfamily
Relative fold expression (RFE) mRNA of AIF (a), CASP-3 (b), GPX1 (c), and HAT (d) in control, leptin-treated, and leptin-melatonin-20 treated (LM20) rats. *P < 0.05, **P < 0.01, leptin versus control; ***P < 0.01, ****P < 0.001, leptin versus LM20.

Concurrent daily administration of melatonin prevented the adverse effects of leptin, particularly when given at a dose of 20 mg kg⁻¹ body weight per day. Melatonin has been reported to prevent gentamycin-induced testicular toxicity in rats. In addition, it has also been shown to have potent protective effects against anticancer drug-induced testicular toxicity, including reduced sperm count and lowered sperm motility. Recent studies have also demonstrated that melatonin prevents oxidative damage and testicular toxicity induced by ochratoxin A, cyclophosphamide, electromagnetic radiation, testicular ischemia–reperfusion, and hypoxia, and it also supports the antioxidant redox system in the testis. More interestingly, leptin-induced increases in O₂⁻ in primary cultured vascular smooth muscle cells were prevented by melatonin. It is, therefore, possible that melatonin reduced the adverse effects of leptin by reducing the level of oxidative stress. Besides functioning as a synchronizer of the biological clock, melatonin is a powerful antioxidant, particularly protecting nuclear and mitochondrial DNA. It scavenges hydroxyl radical (OH·), peroxynitrite anion (ONOO⁻), O₂⁻, nitric oxide radical (NO·), and peroxy radicals. It is also known to enhance the antioxidant activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and glutathione reductase (GR). Besides this, melatonin has been shown to increase GPx protein in the kidney of spontaneously hypertensive rats (SHR), and increase GPx-1 mRNA expression in human chorion, rat liver, and rat brain cortex. Moreover, it also enhances the ability of cells to resist oxidative damage by inhibiting the pro-oxidant nitric oxide synthase. Reproductive systems of different species have binding sites for melatonin, so it seems reasonable to assume that melatonin also exerts its actions through direct interaction with the cells of the reproductive organs.

CONCLUSION

It appears that leptin administration increases the levels of oxidative stress in the testes causing necrotic-like cell death through TNF and JNK pathways. Melatonin prevents these leptin-induced adverse effects on sperm count, sperm morphology, DNA fragmentation, and apoptosis, which might be due to its antioxidant activity. These findings suggest that melatonin might be a useful protective agent...
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