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Melatonin Prevents the Adverse Effects of Leptin on Histone-to-Protamine Transition during Spermatogenesis in Rats

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Abstract

The aim of this study was to examine the effect of melatonin on leptin induced changes in transition of histone to protamine in adult rats during spermatogenesis. Twelve-week old Sprague-Dawley rats were randomized into control, leptin, leptin-melatonin-10, leptin-melatonin-20 and melatonin-10 treated groups with 6 rats per group. Leptin-treated groups received intra-peritoneal injections (i.p.) of leptin daily for 42 days (60 µg/kg body weight). Rats in the leptin and melatonin treated groups were given either 10 or 20 mg of melatonin/day/kg body weight in drinking water. Melatonin-10 treated group received only 10 mg of melatonin/day/kg body weight in drinking water for 42 days. Control rats received 0.1 ml of 0.9 % saline i.p. for 42 days. Upon completion of the treatment, sperm count, morphology and histone-to-protamine ratio estimation was performed. The expression of HAT, HDAC1, HDAC2, H2B, H2A, H1, PRM1, PRM2, TNP1 and TNP2 genes were analyzed using microarray analyses. Data were analyzed using ANOVA. Sperm count was significantly lower whereas the fraction of sperm with abnormal morphology, the ratio of histone-to-protamine transition and the level of HAT, HDAC1, HDAC2, H2B, H2A, H1, PRM1 were significantly higher in leptin treated rats. No significant differences were evident in these between controls and melatonin treated rats. It appears that exogenous leptin administration adversely affects histone-to-protamine transition, upregulates the genes involved in this replacement and these effects are prevented by concurrent administration of melatonin.

Keywords:
Leptin,
Melatonin,
Sperm,
Protamine,
histone

1. Introduction:

Leptin, a 16-kDa protein, is produced and secreted mainly by the adipose tissue. It is a pleiotropic 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 (Fantuzzi et al., 2000; Mantzoros, 2000; Waelput et al., 2006; Sayed-Ahmed et al., 2012). However, a number of recent reports have indicated some adverse effects of leptin on sperm count and morphology. Exogenous administration of leptin to normal rats for 6 weeks was found to significantly decrease sperm concentration whilst increasing the fraction of

morphologically abnormal sperm and increase sperm DNA fragmentation (Haron et al., 2010; Haron et al., 2013; Abbasihormozi et al., 2013; Almabhouh et al., 2014). Although the precise mechanism for this is still unknown, it is also unclear if leptin affects the transition of histone to protamine. Protamines are the most abundant nucleoproteins in mature sperm. During spermatogenesis, histones are first replaced by testis-specific histone variants, which are then replaced by transition proteins. The transition proteins in the condensing chromatin are then replaced with protamines (Carrell et al., 2007). Approximately, 85-95% of histones in the DNA are replaced by protamines, which help provide a tight packaging of the sperm DNA. This compaction of the nucleus and cessation of gene expression helps protect the paternal DNA from damage and mutagenesis (Conwell et al., 2003; Oliva, 2006; Barratt et al., 2010; Rathke et al., 2014). Failure of this could expose the DNA to fragmentation, particularly when the DNA repair process is also temporarily suspended. Abnormalities in histone to protamine transition have been found to be associated with male infertility (Lourdes Mengual et al., 2003; Nasr-Esfahani et al., 2004; Yu et al., 2014). Our recent study showed that, exogenous leptin administration increases DNA fragmentation and 8-hydroxy-2-deoxyguanosine, a DNA marker of oxidative stress (Almabhouh et al., 2014). Altered histone to protamine ratio could make sperm DNA more susceptible to ROS attack and fragmentation. The impact of leptin on these processes has not been reported before and it is possible that leptin either directly or through increased oxidative stress might be influencing these processes resulting in increased DNA fragmentation. This study therefore examined the effect of leptin on the transition of histone to protamine in adult rat sperm.

2. Materials and methods:

Experimental animals:

Twelve-week old male Sprague-Dawley 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 intra-peritoneal injections (i.p.) of leptin once daily for 42 days (60 µg/kg body weight) (Recombinant Rat leptin; purity >95% Biovision USA). Rats in the leptin and melatonin treated group, in addition to leptin, were also given either 10 or 20 mg of melatonin/kg body weight/day (Sigma-Aldrich,) in drinking water for 42 days. Melatonin-10 treated group received only 10 mg of melatonin/ kg body weight/day in drinking water for 42 days. Control rats received 0.1 mL of 0.9% saline i.p. for 42 days. Body weight of control and experimental animals was monitored weekly.

Sample collection:

Upon completion of the treatment, the rats of each group were anaesthetized with diethyl ether in a closed glass chamber. Laparotomy and sample collection were performed and both epididymides and testis were removed. These tissues were immediately washed in 0.9% saline to remove the blood. The epididymides were used for sperm count, morphology and histone to protamine ratio. The testis was stored at- 80°C for further analysis.

Sperm collection:

Sperm from the epididymides were collected according to method used by Haron MN et al. 2010. The epididymis was minced in 2 mL normal saline and filtered through nylon mesh. An aliquot of the epididymal suspension was used for sperm count, percentage of sperm with abnormal morphology and histone to protamine ratio estimation.

Sperm count and morphology:

Sperm count and percentage of sperm with abnormal morphology were estimated using a Makler counting chamber (Sefi Medical Instruments LTD.). The sperm specimen was mixed well and then with the aid of a pipette, a small drop was placed in the center of the disc area of Makler chamber and covered with a glass slip. The drop was allowed to spread onto the entire area of the disc. The number of total and abnormal sperm in a strip of 10 squares was counted. This number represented the concentration of sperm in million per ml. This was repeated on another two strips and then the average was determined and the concentration was expressed in million per ml. The percentage of sperm with abnormal morphology was calculated by counting the number of sperm with abnormal morphology, dividing by the total sperm count and multiplying the product by 100.

Histone to protamine ratio in sperm nuclei:

The ratio of histone to protamine in sperm nuclei was measured using a Sperm Func^{R} Histone Kit, Aniline blue staining method, (Bred Life Science China). Briefly, 5 µL of the sperm suspension were spread evenly onto a glass slide provided in the kit and allowed to dry. The

dried smears were then fixed in 40% methanol for 2 minutes. The slides were then stained with 5% aqueous aniline blue stain mixed with 4% acetic acid for 5 minutes. The slides were then washed in running water and placed into the slide barrel filled with 4% hydrochloric acid for 5 minutes. After that, the slides were washed with tap water and stained with xanthene for 5 minutes. Following that, the slides were washed in running water and allowed to dry. A total of 200 sperm cells were evaluated under optical microscope at 100 x objective. Positively stained sperm (stained blue) had erroneous histone-to-protamine transition. This abnormality in histone transition is defined as the percentage of sperm cells that had elevated histone-to-protamine ratios.

Microarray analysis:

The expression of genes involved in spermatogenesis was determined by microarray analysis. Total cellular RNA was extracted from testes tissue using innuPREP RNA Mini Kit (Analytikjena, Germany) according to the manufacturer's protocol, followed by treatment with DNase (Thermo Scientific). The RNA quality and concentration were assessed. About 200 ng of total RNA were used to prepare amplified cDNA using reagents provided in the Applause WT-Amp plus ST System Kit (Nugen technology, USA). The cDNA was then purified using QIAGEN's MinElute reaction Cleanup Kit. The purified cDNA was measured for concentration and purity. The Encore Biotin Module (Part No. 4200) was used to label the cDNA. Hybridization was then performed on labeled cDNA using GeneChip Hybridization kit (Affymetrix Rat GeneChip St 2) for 18 hour at GeneChip Hybridization Oven 640 (Affymetrix). Immediately following hybridization, the GeneChip arrays were washed and stained using wash and stain kit in an automated GeneChip Fluidic Station 450 (Affymetrix), followed by scanning on a GeneChip Scanner. Data from microarray were analyzed as gene level differential expression by Affymetrix software (ExpressionConsole-1-3-1-64bit and Transcriptome Analysis Console-2-0-64bit). Gene expressions that were up- or down-regulated by at least 2-fold following leptin treatment or leptin-melatonin-20 treatment were compared to control.

Statistical Analysis:

Data were analysed using one way ANOVA with *post hoc* Tukey's analysis contained in SPSS version 20 (IBM, NY, USA) and expressed as mean ± SEM. Statistical significance was accepted at $P<0.05$.

3. Result:

Body weight increased in all rats over the six-week study period (Table 1). However, no significant differences were evident in body weight between leptin, leptin-melatonin-10, leptin-melatonin-20, melatonin-only treated groups and that of the control group.

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

Groups	Body weight (g) Mean ± SEM Day 0	Body weight (g) Mean ± SEM Day 42
Control	345.83 ± 4.56	412.67 ± 6.48
Leptin	344.33 ± 5.02	411 ± 5.16
Leptin-melatonin10 (LM10)	343.5 ± 2.23	410 ± 6.8
Leptin-melatonin20 (LM20)	339.33 ± 2.4	403.67± 1.73
Melatonin 10 (M10)	342.33 ± 1.63	413.33 ± 3.99

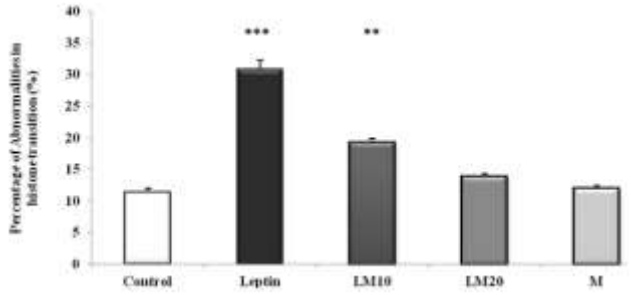
Table 2 *Total Sperm Count and fraction of sperm with abnormal morphology in Leptin and Melatonin Treated Rats*

Groups	Total sperm count ± SEM (Million/mL)	Fraction of abnormal sperm Percentage ± S.E.M (%)
Control	9.9 ± 0.15	3.6 ± 0.3
Leptin	6.1 ± 0.25***	10.9 ± 1.0***
Leptin-melatonin 10 (LM-10)	7.4 ± 0.33** ##	5.2 ± 0.4** ###
Leptin-melatonin 20 (LM-20)	10.1 ± 0.19 ###	5.0 ± 0.18** ###
Melatonin 10 (M-10)	9.8 ± 0.11	4.1 ± 0.2

** *p<0.01*, *** *p<0.001 compared to the control group*
p<0.01, ### p*<0.001 compared to the leptin-treated group*

Sperm count was significantly lower in leptin-only and leptin-melatonin-10 (LM10) treated rats when compared to that in the controls ($p<0.001$ and 0.01 respectively; Table 2). No significant difference was evident in sperm count between control and leptin-melatonin-20 (LM20) and melatonin-10 (M10) treated rats.

The fraction of sperm with abnormal morphology was significantly higher in leptin only treated rats when compared to that in the controls ($p<0.001$; Table 2). No significant differences were evident in the fraction of sperm with abnormal morphology between melatonin-10 (M10), leptin-melatonin-10 (LM10), leptin-melatonin-20 (LM20) and saline treated controls.



***p<0.0, 1 ***p<0.001 compared to control.*

Figure 1 *Fraction of sperm with abnormal histone transition in leptin, leptin-melatonin and melatonin Treated Rats*

No significant difference was evident in the fraction of sperm with abnormal histone transition between control and leptin-melatonin-20 (LM20) and melatonin-10 (M10) treated rats. However, leptin-only treated rats and leptin-melatonin-10 (LM10) treated rats had significantly elevated histone-to-protamine ratios when compared to that in the controls ($p <0.001$ and 0.01 respectively; Figure 1).

Microarray analysis revealed approximately 161 genes involved in spermatogenesis that were differentially expressed in leptin treated rats compared to those in the control rats. The expression of 141 of these genes was up-regulated whereas that of 20 genes was down-regulated. The results showed that genes of basic nuclear protein that are responsible for the nucleosomal structure, such as histone, were up-regulated in leptin treated rats and unchanged in leptin-melatonin-20 rats. Protamine 1 was significantly increased in leptin treated rats while protamine 2 was unchanged in the same rats. In contrast, in the leptin-melatonin-20 group both protamine 1 and 2 were significantly increased compared to those in the control group. The expression of transition protein 1(TNP1) was upregulated in leptin treated rats but transition protein 2 (TNP2) was unchanged. However, both transition proteins TNP1 and TNP2 were upregulated in leptin-melatonin-20 rats. In addition, the expression of histone acetyltransferase (HAT) and histone deacetylase1 and 2 (HDAC1 and HDAC2) was significantly elevated in leptin treated rats and unchanged in leptin-melatonin-20 rats (Table 3).

Table 3 *Expression of histone-protamine Genes Involved in Spermatogenesis in Leptin and Leptin-melatonin-20 Rats*

Gene Description	Gene Symbol	Leptin-treated versus control			Leptin-melatonin-20 versus control		
		Status	Fold change	p-value	Status	Fold change	p-value
Histone linker H1 domain, spermatid-specific 1	Hils1	Upregulated	3.32	0.0132	Unchanged	-	-
H2A histone	H2A	Upregulated	2.59	0.0439	Unchanged	-	-
Histone cluster1	H1t	Upregulated	5.29	0.0012	Upregulated	2.16	0.0054
Histone cluster1 H2b	H2B	Upregulated	2.02	0.0105	Unchanged	-	-
Protamine1	PRM1	Upregulated	2.21	0.0021	Upregulated	2.42	0.0041
Protamine2	PRM2	Unchanged	-	-	Upregulated	2.59	0.0227
Transition protein 1	TNP1	Upregulated	2.79	0.0005	Upregulated	2.61	0.0055
Transition protein 2	TNP2	Unchanged	-	-	Upregulated	2.35	0.0001
Histone acetyltransferase	HAT	Upregulated	2.17	0.0266	Unchanged	-	-
Histone deacetylase1	HDAC1	Upregulated	4.3	0.0172	Unchanged	-	-
Histone deacetylase2	HDAC2	Upregulated	4.07	0.0044	Unchanged	-	-

A number of genes involved in oxidative stress were differentially expressed in the testes tissue of leptin-only treated rat (Table 4). Five of these genes were of antioxidant enzymes including catalase (Cat), glutathione peroxidase7 (Gpx7), glutathione peroxidase 1 (Gpx1), Peroxiredoxin 1 (Prdx1) and glutathione S-transferase pi 1(Gstp1), which were significantly down-regulated in leptin treated rats. These genes were unchanged in LM-20 rats when compared to controls. Cytosolic forms of superoxide dismutase 1 (Sod1) and glutathione s-transferase theta (Gstt4) were up-regulated. Among the up-regulated genes, respiratory

chain reaction enzymes were dramatically up-regulated in leptin treated rats when compared to their expression in LM-20 where they were unchanged (Table 4).

Table 4 *Changes in expression of genes related to oxidative stress*

Gene Description	Gene Symbol	Leptin-treated versus control			Leptin-melatonin-20 versus control		
		Status	Fold change	p-value	Status	Fold change	p-value
Catalase	*Cat*	Down-regulated	-2.2	0.001	Unchanged	-	-
Peroxiredoxin 1	*Prdx1*	Down-regulated	-2.62	0.022	Unchanged	-	-
Glutathione peroxidase 7	*Gpx7*	Down-regulated	-2.27	0.011	Unchanged	-	-
Glutathione peroxidase 1	*Gpx1*	Down-regulated	-2.28	0.009	Unchanged	-	-
Glutathione peroxidase 4	*Gpx4*	Down-regulated	-3.45	0.000	Unchanged		
Glutathione S-transferase pi 1	*Gstp1*	Down-regulated	-2.3	0.014	Unchanged	-	-
Glutathione S-transferase, theta 4	*Gstt4*	Up-regulated	10.5	0.002	Unchanged	-	-
Superoxide dismutase 1, cytosolic form	*Sod1*	Up-regulated	4.37	0.001	Unchanged	-	-
Superoxide dismutase 2, mitochondrial	*Sod2*	Unchanged	-	-	Unchanged	-	-
Superoxide dismutase 3 extracellular	*Sod3*	Unchanged	-	-	Unchanged	-	-
Nuclear factor erythroid 2	*Nfe2*	Unchanged	-	-	Up-regulated	2.49	0.0077
NADH dehydrogenase complex I, assembly factor 3	*Ndufaf3*	Upregulated	3.68	0.015	Unchanged	-	-
Succinate dehydrogenase complex	*Sdh*	Upregulated	2.91	0.000	Unchanged	-	-
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	*Uqcrfs1*	Upregulated	2.87	0.003	Unchanged	-	-
Cytochrome C oxidase assembly factor 3	*Coa3*	Upregulated	4.42	0.025	Unchanged	-	-
COX20 cytochrome C oxidase	*Cox20*	Upregulated	3.41	0.000	Unchanged	-	-
Cytochrome b5 reductase 1	*Cyb5r1*	Upregulated	2.69	0.010	Unchanged	-	-
Ubiquinol cytochrome c reductase core protein 2	*Uqcrc2*	Upregulated	2.67	0.008	Unchanged	-	-

4. Discussion:

The most significant finding of this study is the evidence of elevated histone-to-protamine ratios following six weeks of leptin treatment in the rat (Figure 1). To our knowledge this is the first report on the effect of leptin on histone-protamine transition in sperm and its prevention by melatonin. In addition, leptin treated rats had lower sperm count and higher fraction of sperm with abnormal morphology when compared to that in saline treated controls (Table 2). There was also evidence of up-regulation of respiratory chain enzyme genes and down-regulation of some of the anti-oxidant enzyme genes following leptin treatment (Table 4). Most of the genes involved in histone-to-protamine transition process, including the HAT cluster genes were also up-regulated in leptin treated rats (Table 3). Interestingly, all these effects were absent in rats given concurrent leptin and melatonin treatment.

Decreases in sperm count and increased fraction of sperm with abnormal morphology following leptin treatment have been reported before (Haron et al., 2010; Haron et al., 2013; Abbasihormozi et al., 2013; Almabhouh et al., 2014). Although the precise mechanism for these remains unclear, increased intracellular levels of ROS following leptin treatment in rats has been reported recently (Abbasihormozi et al., 2013). It is therefore possible that increased oxidative stress resulting from leptin administration increases DNA fragmentation, and consequently higher fraction of sperm with abnormal morphology and lower sperm count. The finding that melatonin prevented these suggests that this might indeed be the case (Table 2).

One very important process that takes place during spermiogenesis, is the transition of the sperm chromatin from a nucleosome- to a protamine-based

chromatin in which 85–95% of histones in the DNA are replaced by protamines 1 and 2 (P1, P2). This facilitates the compaction of the nucleus, and in a way, protects the paternal DNA from damage and mutagenesis (Conwell et al., 2003; Oliva, 2006; Barratt et al., 2010; Rathke et al., 2014; Bao and Bedford, 2016). In the present study, the ratio of histone-to-protamine was significantly higher in leptin treated rats compared to that in the controls. Besides that, the expression of histone cluster genes (H1, H2A and H2B) and protamine 1(PRM1) gene was significantly up-regulated in leptin treated rats compared to that in the control (Table 3). This up-regulation might suggest that leptin hastens the histone-protamine transition process. The protamine 2 gene was however not up-regulated. The exact reason for this is unclear. Protamines 1 and 2 are usually expressed in nearly equal quantities. Altered P1/P2 ratios have, however, been associated with increased DNA fragmentation (Garcia-Peiro et al., 2011; Simon et al., 2011). Elevated or reduced P1/P2 ratios have been observed in some infertile men and are often associated with severe spermatogenesis defects (Corzett et al., 2002; Carrell et al., 2007). A reduction in P2 protamine content has also been reported in infertile males (Balhorn et al., 1988; Bach et al., 1990; De Yebra et al., 1993, 1998; Bench et al., 1998). Histone acetylation has been known to play an important role in spermatogenesis. The expression of histone acetyl transferases (HAT) and histone deacetylase (HDAC) was significantly higher in leptin treated rats compared to control and leptin-melatonin-20 treated rats (Table 3). Histone acetylation of lysine residues is regulated by histone acetyltransferases (HAT) to facilitate acetylation whereas histone deacetylases (HDAC) decrease acetylation (Davie, 1998; Kim et al., 2014). Acetylation-associated enzyme activity and sperm DNA fragmentation index, are positively correlated with histone acetyl transferase (HAT) activity (Kim et al., 2014). When histones are hyperacetylated, their affinity to DNA is decreased due to a loss of their positive charge and this makes the chromatin structure more relaxed, permitting transcription to occur (Turner, 1991). When histones are hyperacetylated, histone bound to DNA becomes less tightly compact and more susceptible to damage and DNA fragmentation. The higher expression of the HAT genes suggest that leptin hastens acetylation, making the chromatin structure less compact. Given that leptin is also known to increase ROS production, the presence of increased ROS around chromatin that is less compact could lead to DNA damage and fragmentation. Leptin has been shown to increase superoxide anion production ($O_2^{\cdot -}$) in primary cultured vascular smooth muscle cells (Martínez-Martínez et al., 2014), in aortic endothelial cells (Yamagishi et al., 2001), and cause peroxynitrite-mediated oxidative stress in steatohepatic lesions (Chatterjee et al., 2013). Increased levels of sperm 8-OHdG, a marker of DNA damage due to oxidative stress, and sperm DNA fragmentation following leptin treatment in Sprague-Dawley rats have been reported recently (Almabhouh et al., 2014). Oxidative stress induced by cigarette smoking is also strongly associated with abnormalities in histone-to-protamine transition and with alterations in protamine mRNA expression in human sperm (Hammadeh et al., 2010; Yu et al., 2014).

Concurrent daily administration of melatonin through drinking water prevented the adverse effects of leptin, particularly when given at a dose of 20 mg/kg body weight per day. Melatonin has been reported to prevent gentamycin-induced testicular toxicity in rats (Kim et al., 2013). 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 (Ateşşahin et al., 2006; Ilbey et al., 2009). Recent studies have demonstrated that melatonin prevents oxidative damage and testicular toxicity induced by ochratoxin A, cyclophosphamide, electromagnetic radiation, testicular ischaemia–reperfusion and hypoxia and also supported the antioxidant redox system in the testis (Malekinejad et al., 2011; Koksal et al., 2012; Zepeda et al., 2012; Chabra et al., 2014; Oksay et al., 2012). More interestingly, leptin-induced increase in superoxide anion production ($O_2^{\cdot -}$) in primary cultured vascular smooth muscle cells was reportedly prevented by melatonin (Martínez-Martínez et al., 2014). It is therefore possible that melatonin reduced the adverse effects of leptin on by reducing the level of oxidative stress.

In conclusion, it appears that exogenous leptin administration adversely affects histone-to-protamine transition during spermatogenesis. It also significantly increases the expression of genes involved in replacement of histone to protamine. These effects are prevented by concurrent administration of melatonin.

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منع التأثيرات السلبية الضارة لهرمون اللبتين علي نسبة تغيير الهيستون إلي بروتامين في الحيوانات المنوية للفئران البيضاء من خلال إعطاء هرمون الميلاتونين

كلمات مفتاحية:
اللبتين
ميلاتونين
الحيوانات المنوية
البروتامين
الهيستون

هدفت هذه الدراسة لفحص منع التأثيرات السلبية لهرمون الليبتين على معدل تحول الهيستون إلى بروتامين في الحيوانات المنوية لدى الفئران البيضاء البالغة بواسطة هرمون الميلاتونين, بعد حقن مجموعة من الفئران جرعة مقدارها 60 ميكروجرام لكل كيلوجرام من وزنها بهرمون الليبتين بشكل يومي ولمدة ست أسابيع ومجموعة ثانية أعطيت جرعات من الميلاتونين مقدارها 20 ميليجرام اضافة الى الليبتين ومجموعة ثالثة أعطيت ميلاتونين بجرعة مقدارها 10 ميليجرام اضافة الى الليبتين ومجموعة رابعة أعطيت فقط ميلاتونين ومجموعة خامسة ضابطة بدون حقن. بعد انتهاء فترة التجربة تم قياس عدد الحيوانات المنوية ونسبة الحيوانات المنوية المشوهة ومعدل الهستون الى البروتامين وقياس تغيرات مجموعة من الجينات المشاركة في عملية تكوين الحيوانات المنوية, أظهرت الدراسة انخفاض في عدد الحيوانات المنوية في الفئران المعالجة باللبتين وزيادة في نسبة الحيوانات المشوهة مقارنة مع المجموعة الضابطة كما بينت الدراسة زيادة في نسبة الهيستون الى البروتامين في الفئران المعالجة باللبتين وأظهرت الدراسة أيضاً تتغيرات في التعبير الجيني لمجموعة من الجينات. وختاما، أظهرت الدراسة أن إعطاء هرمون الميلاتونين منع هذه التأثيرات السلبية لهرمون اللبتين على عدد الحيوانات المنوية وشكلها، وانتقال الهيستون إلى بروتامين خلال تكوين الحيوانات المنوية.