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Leptin Adversely Affects The Transition of Histone-to-Protamine during Spermatogenesis in Sprague-Dawley Rats

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Abstract

The aim of this study was to examine the effect of leptin on the histone to protamine ratio in adult rat sperm. Twelve-week old Sprague-Dawley rats were randomized into control and leptin-treated groups with 6 rats per group. Leptin-treated group received intra-peritoneal injections (i.p.) of leptin daily for 42 days (60 µg/kg body weight). 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 measurements were performed. Gene expressions of HAT, HDAC1, HDAC2, H2B, H2A, H1, PRM1, PRM2, TNP1 and TNP2 were analyzed using microarray analyses. Data were analyzed using ANOVA. The results showed that, sperm count was significantly lower among leptin-treated rats compared to control one, whereas the fraction of sperm with abnormal morphology, the histone-to-protamine ratio and the expressions of HAT, HDAC1, HDAC2, H2B, H2A, H1, PRM1 were significantly higher in leptin treated rats. As a conclusion, exogenous leptin administration adversely affects sperm count and morphology, which might be related to alteration in the histone-to-protamine transition during spermatogenesis.

Keywords:
gene expression;
histone; leptin;
protamine;
sperm count.

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 and Faggioni. 2000, Mantzoros, 2000, Waelput. et al. 2006 and 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 while increasing the fraction of morphologically abnormal sperm (Haron et al. 2010, Haron et al. 2013). Increased sperm DNA fragmentation following leptin treatment has been reported recently (Abbasihormozi et al.2013;

Almabhouh et al. 2015 and Almabhouh et al 2016). Although, few studies have been carried out about effect of leptin on sperm count , it is unclear if leptin affects the transition of histone to protamine, which might contribute to the abnormal sperm morphology and count. 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, resulting in compaction of the nucleus and cessation of gene expression (Conwell *et al*. 2003; Oliva, 2006; Barratt *et al*. 2010). Abnormalities in histone to protamine transition have been found to be associated with male infertility (Mengual et al 2003; Nasr-Esfahani et al. 2004, Bolan *et al* 2014). Our recent study showed that, exogenous leptin administration increases DNA fragmentation and the DNA marker of oxidative stress (Almabhouh et al. 2015 and Almabhouh et al 2016). Altered histone to protamine ratio could make sperm DNA more susceptible to 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, consequently resulting in increased DNA fragmentation. This study, therefore, aimed to examine the effect of leptin on the transition of histone to protamine among adult rat sperms.

1. Materials and methods

2.1 Experimental animals

12-week old male Sprague-Dawley rats were randomized into two groups consisting of control and leptin-treated groups with 6 rats per group. Leptin-treated group 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). Control rats received 0.1 ml of 0.9 % saline i.p. for 42 days. Body weights of control and experimental animals were monitored weekly.

2.2 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. The removed tissues were immediately immersed in 0.9 % saline solution for the removal of blood. The epididymides were used for sperm count, morphology and the histone to protamine transition ratio. The testis was stored at -80°C for further analysis.

2.3 Sperm collection

Sperm from the epididymis were collected according to method used by Haron 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 the histone to protamine ratios examination.

2.4 Sperm count and morphology

Sperm count and percentage of sperm with abnormal morphology was determined 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 the Makler chamber. After that, a glass cover slip was placed. The drop was then 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 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 of sperm count and multiplying the product by 100.

2.5 The ratio of histone to protamine 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 prepared sperm suspension were spread evenly onto a glass slide provided in the kit and allowed to dry. Then the smears were

fixed in 40 % methanol for 2 minutes. The slides were then stained with 5 % aqueous aniline blue mixed with 4 % acetic acid for 5 minutes. After this they were washed in running water and then placed into the slide barrel filled with 4 % Hydrochloric acid for elution for 5 minutes. After that, the slides were once again washed with tap water. Then, the slides were stained with xanthene for 5 minutes, following that, the slides were washed with running water and allowed to dry. A total of 200 sperm cells were evaluated under optical microscope at 100x 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 ratio.

1.6 Microarray analysis

The expression of genes involved in spermatogenesis was determined using 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. An amount of 200ng 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 were 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. Then hybridization was applied on label 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 employing an automated protocol on the GeneChip Fluidic Station 450 (Affymetrix), followed by scanning on a GeneChip Scanner. Data from microarray were analyzed as gene level differential expression by Affymetrix softwares (ExpressionConsole-1-3-1-64bit and Transcriptome Analysis Console-2-0-64bit). Expression of at least 2-fold up-regulated or down-regulated in leptin treated or leptin-melatonin-20 treated rats were compared to those of the control ($p<0.05$).

2.7 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 $P<0.05$. The column chart of sperm count concentration, fraction of sperm with abnormal morphology and sperm histone to protamine ratio of control and leptin-treated rats were plotted using Microsoft Excel program version 2013.

3. Results

3.1 Body weight:

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-treated rats and that of the control group.

3.2 Sperm count:

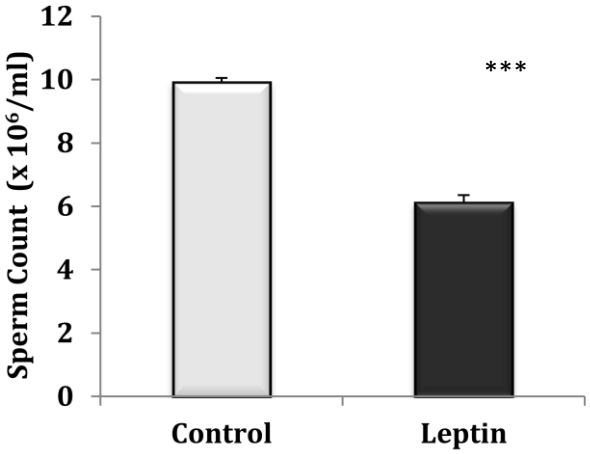
Sperm count was significantly lower in leptin-treated rats when compared to that in the controls ($p <0.001$; Figure 1).

3.3 Sperm Morphology:

The fraction of sperm with abnormal morphology was significantly higher in leptin-treated rats when compared to that in the controls ($p <0.001$; Figure 2).

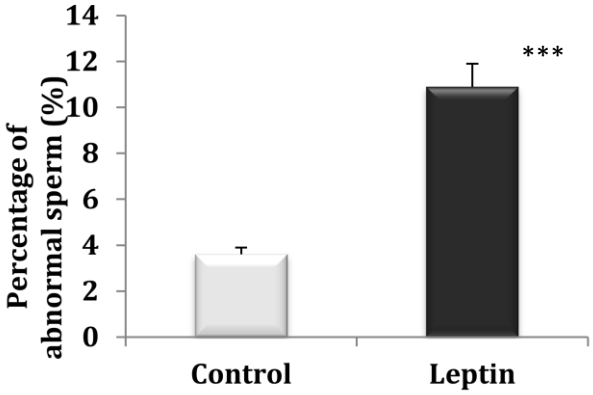
Table 1 ***Body weight of control and leptin-treated rats***

Groups	Body weight (g) ± SEM Day 0	Body weight (g) ± SEM Day 42	*P value*
Control	345.83 ± 4.56	412.67 ± 6.48	0.979
Leptin	344.33 ± 5.02	411 ± 5.16	0.998



*** p<0.001 compared to the control.

Figure 1 ***Sperm Count among control and Leptin-treated Rats***



***p<0.001 compared to control.

Figure 2 ***Fraction of sperm with abnormal morphology in leptin treated and control rats.***

3.4 The ratio of histone-to-protamine:

Mean histone to protamine ratio was significantly higher in leptin-treated rats when compared to that in control group (p<0.001; Figure 3).

3.5 Microarray analysis:

Microarray analysis revealed approximately 161 genes involved in spermatogenesis were differentially expressed in leptin treated rats compared to control rats. The expressions of 141 genes were upregulated and of 20 genes were downregulated. The results showed that genes of basic nuclear protein that are responsible for the nucleosomal structure such as histone were upregulated in leptin treated rats. Protamine 1 was significantly increased in leptin treated rats while protamine 2 was unchanged. Transition protein 1(TNP1) was upregulated in leptin treated rats and transition protein 2 (TNP2) was unchanged. In addition, the expression of histone acetyltransferase (HAT) and histone deacetylase1 and 2 (HDAC1 and HDAC2) were significantly elevated in leptin treated rats compared to that in control rats as shown in Table 2

4. Discussion

The most significant finding of this study is the evidence of elevated histone-to-protamine ratios following six weeks leptin treatment in the rat. However, most genes involved in histone-to-protamine transition process were upregulated in leptin treated rats.

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. 2015 and Almabhouh et al 2016). 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). Leptin has also been shown to increase superoxide anion production (O_{2^-}) 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). We have recently reported increased levels of sperm DNA fragmentation and sperm 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of DNA damage due to oxidative stress, following leptin treatment in Sprague-Dawley rats (Almabhouh et al. 2015 and Almabhouh et al 2016). The DNA damage could lead to apoptosis and eventual reduction in sperm concentration. However, exactly how leptin makes the sperm DNA susceptible to ROS attack is unclear and require further investigations. 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).

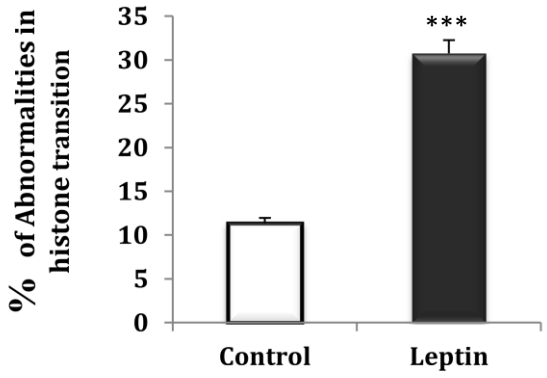
This transition from histone to protamine is also believed to protect the sperm DNA from ROS attack. Altered P1/P2 ratios have been associated with increased DNA fragmentation (Garcia-Peiro et al., 2011; Simon et al., 2011). The impact of leptin on histone-to-protamine transition has not been reported before, and it is possible that leptin either directly or through its effect on the expression of genes involved in this process, or through some other yet unknown mechanism might alter the histone protamine ratio thereby making the sperm DNA more susceptible to ROS attack and fragmentation. Protamines 1 and 2 are usually expressed in nearly equal quantities, but elevated or reduced P1/P 2 ratios have been observed in some infertile men and are often associated with severe spermatogenesis defects (Corzett et al. 2002, Carrell et al. 2007).

The ratio of histone-to-protamine was significantly higher in leptin treated rats compared to that in the control; suggesting an incomplete replacement of histone by protamine. The expression levels of the histone cluster genes (H1, H2A and H2B) and protamine 1(PRM1) gene were significantly higher in leptin treated rats compared to their expressions in the control rats. The present findings demonstrate that leptin can alter the expression of PRM1 to PRM2 mRNA ratio in rat

Table 2 ***Expression of genes involved in spermatogenesis among leptin treated and*** **control rats**

Gene Description	Gene Symbol	Leptin-treated versus control		
		Status	Fold change	p-value
Histone linker H1 domain, spermatid-specific 1	Hils1	Upregulated	3.32	0.0132
H2A histone	H2A	Upregulated	2.59	0.0439
Histone cluster1	H1t	Upregulated	5.29	0.0012
Histone cluster1H2b	H2B	Upregulated	2.02	0.0105
Protamine1	PRM1	Upregulated	2.21	0.0021
Protamine2	PRM2	Unchanged	-	-
Transition protein 1	TNP1	Upregulated	2.79	0.0005
Transition protein 2	TNP2	Unchanged	-	-
Histone acetyltransferase	HAT	Upregulated	2.17	0.0266
Histone deacetylase1	HDAC1	Upregulated	4.3	0.0172
Histone deacetylase2	HDAC2	Upregulated	4.07	0.0044

testes cells. The mechanism by which leptin-induces the abnormality in protamine and histone transition is unclear. A reduction in P2 protamine content has been reported in infertile human males (Balhorn et al, 1988; Bach et al, 1990; De Yebra et al, 1993, 1998; Bench et al, 1998).



****p<0.001 compared to control.*

Figure *3 Sperm histone to protamine ratio in control and leptin-treated Rats*

Although the markers of oxidative stress were not estimated in this study but findings from earlier studies suggest that leptin increases oxidative stress and this might also interfere with the histone to protamine transition significantly affecting the protamine replacement process.

Oxidative stress induced by cigarette smoking has also been strongly associated with abnormalities in histone-to-protamine transition and with alteration in protamine mRNA expression in human sperm (Hammadeh et al. 2010; Bolan et al. 2014).

Our findings revealed that, the expression of histone acetyl transverses (HAT) and histone deacetylase (HDAC) were significantly higher in leptin-treated rats compared to control rats. Histone acetylation has been known to play an important role in spermatogenesis (Kim et al. 2014). Histone acetylation of lysine residues, clustered at the amino-terminal end of core histones, is regulated by HAT to facilitate acetylation and HDAC to decrease acetylation (Davie 1998, Kim et al. 2014). A recent study demonstrated a significant relationship of acetylation-associated enzyme activity and sperm DNA fragmentation index and sperm DNA fragmentation index was positively correlated with HAT activity (Kim et al. 2014). When histones are hyperacetylated, their affinity to DNA is decreased through a loss in their positive charge, and this makes the DNA chromatin structure more relaxed, thereby permitting active transcription occurs (Turner, 1991). However, when histones are hyperacetylated, histones bound to DNA become less tightly compacted making the DNA more susceptible to ROS attack and fragmentation. The impact of leptin on the upregulation of HAT gene and hyperacetylation coupled with its well-known ROS producing properties might lead to increased DNA fragmentation. Our findings of an upregulation of HAT gene in leptin treated rats and increased DNA damage strongly support this idea.

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. Coupled to its ROS producing actions it leads to sperm DNA damage and fragmentation, which then leads to abnormal sperm morphology and sperm apoptosis.

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تأثير هرمون الليبتين على معدل تحول الهيستون إلى بروتامين في الحيوانات المنوية لدى الفئران البيضاء البالغة

هدفت هذه الدراسة لفحص تأثير هرمون الليبتين على معدل تحول الهيستون إلى بروتامين في الحيوانات المنوية لدى الفئران البيضاء البالغة، بعد حقن مجموعة من الفئران جرعة مقدارها 60 ميكروجرام لكل كيلوجرام من وزنها بهرمون الليبتين بشكل يومي ولمدة ست أسابيع , مع مجموعة اخرى 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 أيضا تغيرات في التعبير الجيني لمجموعة من الجينات. وختاما، أظهرت الدراسة أن هرمون اللبتين يؤثر سلبا على عدد الحيوانات المنوية وشكلها، وهذه التغيرات قد تكون ذات صلة للتغير في انتقال الهيستون إلى بروتامين خلال تكوين الحيوانات المنوية .