Effect of Visfatin on Testosterone Hormone Level in Chronic Restraint Male Albino Rats

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Abstract

Background: Stress has been linked in many studies with infertility and hormonal irregularities. Visfatin is a novel adipocytokine which has been proven to decrease in chronic stress in rats.

Aim of Study: This study aimed at evaluation of the effect of visfatin in modulation of the complications of chronic restraint on gonadal functions.

Material and Methods: 32 male albino rats randomly allocated to four equal groups, group (I): Included rats fed a normal diet (control group) group (II): Included rats fed a normal diet for 2 months then received a single intraperitoneal 500pmol visfatin injection group (III): Included immobilized rats (4hr/day) for 2 months and group (IV): Included immobilized rats (4hr/day) for 2 months then received a single intraperitoneal visfatin injection at a dose of 500pmol. After the last visfatin dose blood samples were collected and examined for testosterone, LH and FSH. Then laparotomy was conducted to dissect the right testis for histopathological studies.

Results: The restraint stress has inhibitory effects on male testosterone, LH, FSH and testicular tissues in rats, marked deterioration of gonadal functions in immobilization stress group (group III), a significant decrease ($p<0.001$) in serum testosterone, LH, sperm count and right testicular weights, marked deterioration in the gonadal histoarchitecture, but, there was a non-significant change in serum FSH. Exogenous visfatin administration in (group IV) significantly increased serum testosterone ($p<0.01$), LH ($p<0.001$), FSH ($p<0.01$), and sperm count ($p<0.001$) than group III, and markedly improved the gonadal histoarchitecture. Group II demonstrated results similar to group III when compared to the control group.

Conclusion: Visfatin plays a protective role against chronic stress-induced gonadal dysfunction via maintaining testicular steroidogenesis of Leydig cells.

Key Words: Visfatin – Chronic stress – Testicular function.

Introduction

CHRONIC stress is a condition that is induced by any stressor for a long period. Stress affects the physiological functions of the central nervous, cardiovascular, digestive, and neuroendocrine systems, as well as the reproductive system. Biochemical analysis has shown that the increased corticosterone and blood glucose levels are major markers of stress conditions [1].

Many reports have demonstrated that sexual disorders and infertility could result from psychological and physiological stresses [2,3].

Regarding males, suffering stress may cause a significant reduction in the levels of testosterone, gonadotropin-releasing hormone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) [2,4,5], which could hinder the quality of sperms [5-7]. Additionally, they could also endure Leydig cell and seminiferous tubule damages [5,8,9].

Many reports have explained the downregulation of testosterone production which occurs in acute stress events and attributed it to the decreased level of steroidogenic proteins and enzymes such as Steroidogenic Acute Regulatory (StAR) protein and cytochrome P450 side-chain cleavage (CYP11A1) enzyme [10-12].

However, changes in sperm physiology and other testicular markers in conditions of stress require further explanation.

Fukuhara et al., [13] were the first to describe visfatin in 2005, its effect is similar to insulin as it decreases the serum glucose level via binding to insulin receptors, yet, does not compete with it as it binds to different sites. Its level rises after
ingesting high-fat food which suggests that it serves a major role in the diet or obesity-induced insulin resistance [14]. It has been reported that visfatin significantly increases testosterone production [15], but its role in testicular steroidogenesis still vague.

Thus, this study aimed at evaluation of the effect of visfatin in the modulation of the complications of chronic restraint on gonadal functions.

Material and Methods

A group of 32 albino male rats (200-250g each) were obtained from the Animal House of Faculty of Veterinary Medicine, Zagazig University and kept in steel wire cages (8 rats per cage), with air-conditioned room and adjusted lights at 12 hours light/12 hours dark cycle and temperature at 21-24ºC, food and water ad libitum was used for their nourishing.

They were randomly allocated to four equal groups:

1- Group I (control): Included 8 rats allowed to move freely in their cages without distribution nor stressors.

2- Group II (visfatin): Included 8 rats not subjected to stressors and received 500pmol single intraperitoneal visfatin injection (i.p) (Lyophilized without any additives, Sigma Aldrich Co. USA) [15] after 2 months from the onset of the experiment.

3- Group III (immobilization stress): Included 8 rats individually exposed to restraint sessions in special animal restraining metal cages adjustable to fit each rat. The restraining session involved placing the rats in the prone position at room temperature, 4h/day, 6 days/week for two months [4].

4- Group IV (visfatin + immobilization stress): Included 8 rats, exposed to similar restraining circumstances as group III and at the end of the restraining period (2 months) received 500pmol single intraperitoneal visfatin injection (i.p).

An approval was obtained for the study procedure from the Institutional Research Board of Faculty of Medicine, Zagazig University, and the study followed the guidelines for the care and use of research animals. Experiments were performed between 9:00AM and 4:00PM (from the 2nd of January to 10th of March 2018).

Blood collection:

Clean plastic centrifuge tubes were used to collect the blood samples (8ml/rat) at the end of the experiment then left till clotted. Samples were then centrifuged for 15 minutes at a speed of 3000 rpm to separate the serum to be then collected by automatically operating pipettes with fine tips and kept at –20ºC till needed for analysis.

Biochemical analysis:

1- Rat Soluble visfatin, (recombinant): From Alexis Biochemicals, Axxora, USA.


4- Estimation of serum FSH level: The technique of Rebar et al., (1982) was adopted using rat follicle-Stimulating Hormone (FSH) enzyme-linked immunosorbent assay kit: (Catalog Number: 2011-11-0183, Shanghai Sunred Biological Technology, China).

Postmortem examination:

The right testis of each animal was dissected then weighed, postmortem, by one hand and the other hand was used for a careful fat dissection [16].

Spermatic parameters analysis:

The epididymis of each testis was subjected to a dissection, as well as mincing in 2ml of Hank's Buffer Salt Solution (HBSS) at 37ºC, [17], then incubated for five minutes at 37ºC, then the standard hemocytometric technique was used for the analysis of the cauda epididymis sperm.

The White Blood Cell pipette (WBC pipette) was used to draw, up to the 0.5 mark, of the epidymal fluid and, up to the 11 mark of the semen diluting fluid (sodium bicarbonate 5g, formalin 1ml, distilled water 99.0ml), then both components were mixed thoroughly. One drop was placed in the hemocytometer chamber and the hemocytometer was kept in a humid environment (wet chamber) for one hour to allow the sperms to settle. Then the light microscope, at 400X, was used to count the sperms in the appropriate squares of the hemocytometer after the incubation. Sperm count = No. of spermatozoa counted X dilution factor X volume factor/No. of areas counted was used to
calculate the number of spermatozoa/ml fluid which refers to the sperm concentration [18].

Histopathological examination:

After collection of blood samples for hormonal analysis, each rat was subjected to a laparotomy to obtain the right testis for subsequent weighing and histopathological assessment as follows:

First, a careful removal of the tunica vaginalis followed by dissection of the testis, to be followed by cleaning using cold physiological saline to remove blood and any adherent tissue. Then a fixation was performed in 10% formaldehyde in fresh alcoholic bouin's fluid for 8 hours, then the sample was subjected to processing and embedding in paraffin wax, then sectioning at 5 µm thickness, and staining in hematoxylin-eosin. The light microscope was used to examine the sections to evaluate the histological features. The technique of [19,20] was adopted for the histological assessment.

Statistical analysis:

SPSS program (Version 18 for windows) (SPSS Inc. Chicago, IL, USA) was used for statistical analysis and the obtained quantitative variables were expressed as mean ± SD. Results of all groups were statistically compared using the One Way Analysis of Variance (ANOVA) then the LSD test. p-value <0.05 was considered statistically significant.

Results

Results showed that immobilized stressed male rats had a lower level of testosterone and LH than the control group (I) (p<0.001), the difference was significant. Upon intake of visfatin, testosterone, LH, and FSH significantly increased in (group II) more than group I, (p<0.01, p<0.001 and p<0.05 respectively) moreover, group IV demonstrated a significantly higher testosterone, LH, and FSH than group III (p<0.01, p<0.001 and p<0.01) (Table 1).

Regarding the epididymal sperm count and right testicular weight, immobilized stressed male rats (group III) had a significantly lower count and weight than the control group (p<0.001). Upon intake of visfatin, in groups (II & IV) both parameters were significantly improved (p<0.001) (Table 1).

Concerning, histopathological results, immobilized stressed male rats (group III) had irregular seminiferous tubules with atrophic walls, low count of sperm cells, and edematous interstitial tissue Fig. (1C). On the other hand, group II had highly regular seminiferous tubules with increased spermatogenesis at the level of spermatocytes Fig. (1B), group IV Fig. (1D) showed a markedly improved testicular histoarchitecture.

Table (1): Serum Testosterone, LH, FSH levels & epididymal sperm count and right testicular weights in rats of all studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(N=8)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum testosterone levels (ng/ml):</td>
<td></td>
<td>3.31±0.42</td>
<td>3.98±0.29</td>
<td>1.47±0.34</td>
<td>2.03±0.35</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p&lt;0.01 a</td>
<td>p&lt;0.001 a,b</td>
<td>p&lt;0.001 a,b,p&lt;0.01 c</td>
<td></td>
</tr>
<tr>
<td>Serum LH levels (µIU/ml):</td>
<td></td>
<td>1.44±0.08</td>
<td>2.05±0.15</td>
<td>1.13±0.12</td>
<td>1.37±0.11</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p&lt;0.001 a</td>
<td>p&lt;0.001 a,b</td>
<td>NSa, p&lt;0.001 b,c</td>
<td></td>
</tr>
<tr>
<td>Serum FSH levels (µIU/ml):</td>
<td></td>
<td>0.23±0.09</td>
<td>0.48±0.07</td>
<td>0.16±0.02</td>
<td>0.26±0.06</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p&lt;0.05 a</td>
<td>NSa, p&lt;0.001 b</td>
<td>NSa, p&lt;0.001 b,p&lt;0.01 c</td>
<td></td>
</tr>
<tr>
<td>Epididymal sperm count (millions/ml):</td>
<td></td>
<td>40.50±4.37</td>
<td>61.12±9.09</td>
<td>27.87±3.72</td>
<td>39.50±4.72</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p&lt;0.001 a</td>
<td>p&lt;0.001 a,b</td>
<td>NSa, p&lt;0.001 b,c</td>
<td></td>
</tr>
<tr>
<td>Right testicular wt (gm):</td>
<td></td>
<td>1.28±0.09</td>
<td>1.56±0.10</td>
<td>0.68±0.14</td>
<td>1.15±0.14</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p&lt;0.001 a</td>
<td>p&lt;0.001 a,b</td>
<td>p&lt;0.05 a,p&lt;0.001 b,c</td>
<td></td>
</tr>
</tbody>
</table>

a: VS group I.  
c: VS group III.  
b: VS group II.  
NS: Non-Significant.
Histopathological studies:

Fig. (1): Light photomicroscopic picture of right testis tissue stained by hematoxylin and eosin (H & E) compared between groups. (A) Showing regular seminiferous tubules with advanced stages of cells spermatogenesis and normal intertubular gaps in the control group. (B) Showing highly regular seminiferous tubules and an increased spermatogenesis at the spermatocytes (arrow) level in visfatin treated group. (C) Showing irregular seminiferous tubules with atrophic walls and low count of sperm cells (arrow) as well as edematous interstitial tissue (pyramid) in immobilization stress. (D) Showing nearly equal seminiferous tubules in different stages of spermatogenesis (arrow). There is no edema in interstitial tissue (pyramid) in visfatin treated immobilization stress group (viewed under low power X200).

Discussion

It has been shown that many aspects of the body physiological functions are influenced by many types of stress such as immobilization which triggers alterations in reproductive functions [21]. Visfatin is an adipokine, excessively produced in visceral fat exhibiting insulin-like functions as it binds to the insulin receptors, which are located on Leydig cells yet at different sites than those belonging to the insulin [13,22,23]. Since insulin enhances testosterone synthesis and visfatin has an insulin-like action, thus visfatin could also increase testosterone level. Therefore, the target of the study in hands was to evaluate the effect of visfatin in the modulation of the complications of chronic restraint on gonadal functions [13,23-25].

Based on results of the current study, the serum level of testosterone, LH, the number of sperms in the epididymis as well as the weight of the testis showed a significant reduction in immobilized stressed rates.

Similar studies have evaluated the consequences of various stress types on the fertility of both humans and animals and revealed that such stressors inhibit the testis and result in a reduction of the serum testosterone [21,26,27]. Additionally, other studies revealed another mechanism of testosterone reduction in such cases which involves an inhibition of LHRH production and secretion from the hypothalamus which results from the depression of the LH serum level as a result of the stress [28]. Moreover, the aforementioned downregulation of the Hypothalamic-Pituitary-Gonad (HPG) axis could be caused by Corticotrophin-Releasing Factor (CRF) and endogenous opioids, mainly \( \beta \)-endorphins that are produced by the hypothalamus as a result of exposure to stress [29].

In addition, CRF and \( \beta \)-endorphins have been proved to affect the HPG axis via inhibition of hypothalamic production of LH-RH [30], depression of pituitary LH [31], and direct inhibition of testosterone formation in Leydig cells [32], which eventually lower the serum testosterone level.
Polyunsaturated fatty acids are abundant in the testicular membrane rendering it vulnerable to oxidative stress [46], several studies have proved the drawbacks of the free radicals on testicular steroidogenesis [38] such as that Nitric Oxide (NO) free radicals which have been implicated in the stress-induced depression of testicular steroidogenesis and testosterone level in stressed immobilized rats [37,46,47].

[47] conducted a study on stressed immobilized rats and found that they suffered a significant reduction of the testicular 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/lyase (P450 C17) and NADPH-P450 reductase activities.

Additionally, [43] assessed testicular 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase in rats exposed to swimming exercise-induced oxidative stress and reported a significant downregulation of their activity.

On the contrary to the results of the study in hands, [48] reported that the serum LH did not decrease in stressed rats, and [19,49] reported that stress does not affect the reproductive function.

The serum FSH did not significantly change in the stressed immobilized rats of the present study and this could be owed to the short duration of the study.

According to the current study, an evident association was noticeable between visfatin administration and male gonadal functions demonstrated as a significant increase in the serum testosterone, LH, FSH, testicular weight & and the number of sperms in the epididymis in both visfatin treated and immobilization stress groups compared to controls (I).

Similarly, [50] demonstrated in their study that the serum LH, FSH significantly increased followed by an increased release of testosterone from testicular Leydig cells. Also, [51] reported a similar finding in their study as they proved the insulin-like action of visfatin as it increases the serum LH and FSH and subsequently the testosterone level. Such effect could be served through two pathways, either directly via stimulation of testicular Leydig cells or indirectly via stimulation of the pituitary gland. The process of testosterone production is mediated mainly by pulses of LH, also, steroidogenesis which is conducted in Leydig cells is mediated locally by circulating hormones, growth factors, and cytokines [52].
The status of the Hypothalamic-Pituitary-Gonadal (HPG) axis is reflected in the level of the serum testosterone, a plethora of stressors could lower the serum level of LH which in turn decreases the serum testosterone via downregulating the hypothalamic synthesis and secretion of LHRH [28]. Such stressors could also impair the functions of Leydig cell, specially steroidogenesis as a result of decreased hormonal levels and cytokines locally in the target cells and in fat cells [28,35].

[15] demonstrated in their study that the secretion of testosterone from Leydig cells is directly proportionate to the level of LH, as they incubated these cells with variable doses of LH [52,53] also reported a similar finding.

Visfatin has a direct stimulatory effect on steroidogenesis Leydig cell and such role is served specifically at the step before the synthesis of pregnenolone. Such steps involved the transportation of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, and the use of cholesterol by cytochrome P450 side-chain cleavage enzyme (Cyt P450 scc). Visfatin cannot increase 25-hydroxycholesterol stimulated steroidogenesis [15].

[15] reported that visfatin significantly increased the testosterone synthesis and release from Leydig cells in the presence of LH 1,000 rjg.

Although, the intracellular mechanism of visfatin action is still vague, several researchers [54,55] have stressed upon the enzymatic mechanisms of testosterone production in the Leydig cell. Both protein kinase C (PKC) and protein kinase A (PKA) blocker downregulate the synthesis of testosterone, such effect is heightened when they are used in conjunction with visfatin which perform its action mainly via Ras/Raf1 kinase system and less through PKC and PKA system of enzymes [15].

Histopathological examination of the stressed immobilized group (III) of the current study demonstrated irregular seminiferous tubules with atrophic walls and low count of sperm cells as well as edematous interstitial tissues. Upon visfatin intake, testicular histoarchitecture significantly improved in both visfatin and immobilization stress treated groups.

Similarly, [15] demonstrated an enhancing action of visfatin on testosterone production which in turn stimulates spermatogenesis in most seminiferous tubules of the treated animals in comparison to their controls.

**Conclusion:**
Visfatin has a protective effect against immobilization stress-induced gonadal dysfunction, such effect is achieved via maintaining gonadal hormonal function through indirect and/or direct effect on the gonads. More investigations are essential to unveil the pathogenesis of these effects and to provide a novel therapy for stress-induced the reproductive disorders.

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تأكير الفسفاتين على مستوى هرمون التستوستيرون
في الجزران البيضاء ذات التوتر العصبي المزمن

خلفية البحث:
قد تم البحث بين التوتر العصبي المزمن في العديد من الدراسات في الغدد sayıء والاضطرابات الدماغية، وقد اكتشف بعض أن هرمون الفسفاتين يعتبر من الهورمونات التي تساعد في خفض التوتر المزمن في الجرزان، ولكن دوره في تنظيم وظائف الغدد التناسلية غير معروف، لهذا صممت هذه الدراسة لتعزيز تأثير هذا الهرمون في الحفاظ على الغدد التناسلية من الآثار السلبية الناتجة عن التوتر المزمن.

مواد وطرق البحث:
أجريت هذه الدراسة على عدد إثنيان وثلاثين من ذكور الجرزان البيضاء البالغة، والتي تم تقسيمها عشوائياً إلى أربع مجموعات متساوية:
- المجموعة الأولى: تم تغذيتها على الغذاء الطبيعي لمدة شهرين.
- المجموعة الثانية: مجموعة دراسة تاثير هرمون الفسفاتين فقد قامنا بتغذيتهاipelًا، الطبيعي لمدة شهرين ثم إعطاؤها هرمون الفسفاتين (10 ديبيكروم) بالفواكه البريتيتي.
- المجموعة الثالثة: مجموعة دراسة تأثير التوتر المزمن، فقد قمنا بتحديها عن طريق منع الحركة 4 ساعات متابعة يومياً لمدة شهرين.
- المجموعة الرابعة: دراسة تأثير هرمون الفسفاتين في الحفاظ على الغدد التناسلية من الآثار السلبية الناتجة عن التوتر المزمن، في هذه المجموعة قمنا بمنع الحركة عن الجرزان 4 ساعات متابعة يومياً لمدة شهرين ثم إعطاؤها هرمون الفسفاتين (10 ديبيكروم) بالفواكه البريتيتي.

وقد أسفرت نتائج هذا البحث:
- حدوث تغييرات كبيرة إحصائية في مستوى هرمون التستوستيرون، وهرمون اللوبتي، وعدد الحيوانات النموية بالبربخ، وزن الخصى اليمنى في المجموعة الثالثة، وكذلك حدوث تغير نلحوظ في حجم وشكل أنياب الخصية وذلك عند فحصها مجهرياً.
- كما أوضحنا نتائج أن الحقن بالفسفاتين في المجموعة الرابعة أدى إلى حدوث زيادة ذات دالة إحصائية في مستوى هرمون التستوستيرون، وهرمون اللوبتي، والهرمون المحفز لاجريات المبيض، وعدد الحيوانات النموية بالبربخ، وزن الخصى اليمنى عند مقارنتها بالمجموعة الثالثة.
- كما أن الحقن بالفسفاتين في المجموعة الثانية أدى إلى تغيرات ملحوظة لما تم ذكره سابقاً في المجموعة الرابعة عند مقارنتها بالمجموعة الأولى المغذية بالغذاء المعتاد.

المستخلص من البحث:
- هرمون الفسفاتين له دور إيجابي ملحوظ في حماية الخصيتين من الأضرار الناتجة عن التوتر العصبي المزمن
- وذلك عن طريق زيادة هرمونات المحفزة للغدد الجنسية.
- ومن نتائج هذا البحث يمكن أن نوصي بعمل المزيد من الدراسات لبحث التأثير العلاجي لهرمون الفسفاتين لحالات الخلل في وظائف الغدد التناسلية الناتجة عن التوتر العصبي المزمن.