Effect of Ghrelin Antagonist on Serum Testosterone and Luteinizing Hormone in Obese Type 2 Diabetic Mice

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Abstract

Background: To determine the effect of ghrelin antagonist (D-Lys6) on plasma glucose, insulin resistance, serum testosterone and luteinizing hormone levels, on obese and type 2 diabetic mice.

Methods: Fifty healthy BALB/c mice were divided into five groups. Mice in group II to V were fed high-fat diet for 4 weeks followed by intraperitoneal (IP) injection of streptozotocin to group IV and V to induce type 2 diabetes mellitus. Group I served as control. Groups II and III were obese and group IV and V were diabetic. Groups III and V were injected ghrelin antagonist IP for 6 days. Terminal intracardiac blood extraction was done and samples were analyzed for fasting plasma glucose, serum insulin, testosterone and luteinizing hormone. Insulin resistance was calculated by homeostatic model assessment for insulin resistance.

Results: Plasma glucose level and insulin resistance decreased and serum testosterone and luteinizing hormone levels increased after using ghrelin antagonist in group III and V.

Conclusion: Glucose level and insulin resistance were significantly decreased in obese and type 2 diabetic mice after ghrelin antagonist administration. Whereas serum testosterone and luteinizing hormone levels were increased.

Key Words: Ghrelin antagonist, testosterone, luteinizing Hormone, obese Type 2 Diabetic Mice

Introduction

Obesity is considered as the epidemic of the modern world. 1 The incidence of obesity has doubled in the past twenty years, which may be due to altered eating habits and sedentary life style. 2 It not only affects the quality of life by decreasing the general body performance, but also leads to the development of many metabolic disorders including diabetes mellitus, insulin resistance, fatty liver disease and atherosclerosis. 3

Type 2 diabetes mellitus is characterized by hyperglycemia, hyperinsulinemia and insulin resistance. More than 80 % cases of type 2 diabetes mellitus are obese. 4 Obesity leads to a decrease in expression and translocation of glucose transporter type 4 (GLUT4) in adipocytes. In addition there is also loss of tyrosine phosphatase activity leading to a loss of 80-90% of its function. 5 Normalization of body mass index leads to reversal of all these mechanisms leading to an improvement in efficacy of blood glucose control mechanisms. 6

It has been found that obesity directly and indirectly affects the male reproductive functions, leading to a decrease in the secretion of male sex hormone i.e. testosterone. 7 A variety of cytokines, adipokines and free fatty acids are secreted from adipocytes which directly affects the plasma androgen levels. 8 The quantitative and qualitative efficacy of Leydig cells is also decreased. There is decrease in the expression of glucose transporter type 3 (GLUT3) on Leydig cells. In addition there also decrease in its tyrosine phosphorylation activity leading to loss of its function. 9 Obesity may also affect male reproductive system indirectly via insulin resistance. Insulin resistance and plasma testosterone levels are found to be in negative correlation with each other. 10 The mentioned effects may also be exerted through ghrelin hormone which is increased in obesity and type 2 diabetes mellitus. 11

Ghrelin is a 28-amino acid peptide hormone was discovered in 1999 from the stomach extracts of rats. 12 It is a hunger stimulating hormone secreted from stomach and pancreas. Ghrelin receptors (G protein-coupled receptors), are expressed in a number of tissues including pituitary, stomach, intestine, pancreas, thymus, gonads, thyroid, and heart. 13 The wide expression of ghrelin receptors suggests that this hormone has diverse functions in different tissues. Ghrelin decreases the secretion of insulin from beta cells of pancreas in response to hyperglycemia (by decreasing the influx of calcium in the beta cells of pancreas secondary to hyperglycemia), leading to an increase in the plasma glucose levels. 14 D-Lys6 GHRP6 (His-D-Lys-Trp-D-Phe-Lys-NH2) acts as ghrelin receptor antagonist. It acts on ghrelin receptor GHS-
R1a and has been used in numerous studies. Administration of ghrelin receptor antagonist (D-Lys³)
in normal and obese mice, leads to an increase in the secretion of insulin accompanied with decrease in
blood glucose levels.15,16

Ghrelin acts as a negative modulator of male reproductive system. It causes a decrease in number of
leydig and sertoli cells in rats. 17 The high level of ghrelin is associated with a decrease in plasma
testosterone and gonadotropin hormones (leutinizing hormone and follice stimulating hormone).18 Ghrelin
also acts upstream in hypothalamo- hypophyseal gonadal axis and reduces the pulse frequency of
gonadotropin-releasing hormone (GnRH). 19 Ghrelin antagonist improves testosterone production in obese
mice. 20 The mechanism involved is not clear. Ghrelin antagonist may act via luteinizing (LH) which in turn
increases the secretion of testosterone. The purpose of this study was to identify the site of action of ghrelin
antagonist in the hypophysial-gonadal axis, which is of clinical significance in obese and diabetic human male
reproductive health.

**Materials and Methods**

This study was conducted at Department of Physiology, Army Medical College, Rawalpindi, in
collaboration with National Institute of Health (NIH), Islamabad, Pakistan. The study was approved by
animal ethics committee of Army Medical College, NUST and all animals received humane
care.Streptozotocin (STZ) and ghrelin antagonist were purchased from Bioplus Fine Research Chemicals and
Sigma Chemicals, USA respectively. Glucose was measured by GOD-POD method. Enzyme linked
immunosorbent assay (ELISA) was used to measure serum insulin, serum testosterone and serum LH
levels.

Five weeks old, 50 healthy BALB/c male mice were purchased from National Institute of Health,
Islamabad. An average weight of mouse was 28.07 g ± 0.1. Mice were divided into five equal groups. They
were given free access to food and water. The room was well ventilated with controlled temperature range of
20-22°C and 12 hours light and dark cycles were maintained. After acclimatization the animals were
randomly divided into five groups. Group 1 (control) was fed normal pellet diet, while group II-V were
given high fat diet ad libitum (to induce obesity and insulin resistance) for 4 weeks. After 4 weeks, group
IV and V were given intraperitoneal injections of streptozotocin 50mg/kg body weight for 5 days, as
previously described to induce type 2 diabetes. STZ
induces rapid and extensive degradation of cellular deoxyribonucleic acid (DNA) of beta cells of
pancreas.21,22 All the other groups were injected intraperitoneally with normal saline. Tail vein blood
samples were used to measure (fasting) blood glucose levels and to confirm diabetes. The blood glucose
level >252 mg/dl was taken as the cut off value for confirming diabetes mellitus. 22 This was followed by
intraperitoneal injection of ghrelin antagonist 10 μmol/kg for 6 days in 9th week 23 to group III and V.
On 62nd day, terminal blood sample was collected by intracardiac puncture. 24 The samples were stored at -
20°C for further analysis. The statistical significance of differences across the groups was determined by
applying analysis of variance Post-hoc (ANOVA) followed by Tukey’s HSD to find the difference in
various pairs of groups. P value < 0.05 was considered significant.

**Results**

There was no significant difference in body weight of mice at the start of study (Table 1). The average
weight of the mice was 28.07g ± 0.10. There was continuous weight gain by the mice throughout the
experiment. Weight of the mice in obese group treated with ghrelin antagonist (group III) were
significantly reduced compared with obese group II (p =0). Ghrelin antagonist treated diabetic group (group
V) mice showed significant reduction in weight as compared with diabetic group IV (p = 0).

**Table1. Weight of BALB/c mice at start and end of experiment**

<table>
<thead>
<tr>
<th>Weight of mice in grams</th>
<th>Group I Control</th>
<th>Group II Obese</th>
<th>Group III Obese with ghrelin antagonist</th>
<th>Group IV Diabetic</th>
<th>Group V Diabetic with ghrelin antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of experiment</td>
<td>28.1± 0.27</td>
<td>28.09± 0.45</td>
<td>28.18± 0.21</td>
<td>27.91± 0.38</td>
<td>28.07± 0.38</td>
</tr>
<tr>
<td>End of Experiment</td>
<td>32.81± 1.56</td>
<td>39± 0.68</td>
<td>36.7± 0.62</td>
<td>41.2± 1.48</td>
<td>37.21± 0.37</td>
</tr>
</tbody>
</table>

There was no significant difference in the blood glucose level of different groups at the start of the
experiment (Table 2). After 8 weeks of study i.e. on
day 55, fasting blood glucose levels were significantly
high in group IV and group V compared with the
control (group I) (p = 0). There was no significant
difference between other groups. At the end of the
experiment blood glucose level in terminal blood
samples showed that group II was significantly raised
compared with the control and group III (p = 0) while
it was significantly low compared with group IV and group V (p = 0.0). Group IV was significantly high compared with Group I and V (p = 0). Blood glucose level at the end of the experiment was significantly higher in Group V compared with the control (p = 0). Ghrelin antagonist significantly reduced the blood glucose levels in group III (p = 0) and Group V (p = 0) compared with group II and group IV respectively.

Table 2. Fasting blood glucose, insulin and HOMA IR levels in BALB/c mice

<table>
<thead>
<tr>
<th>Blood parameters</th>
<th>Group I (n = 10)</th>
<th>Group II (n = 10)</th>
<th>Group III (n = 10)</th>
<th>Group IV (n = 10)</th>
<th>Group V (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Blood glucose level (mg/dl)</td>
<td>97.2 ± 11.87</td>
<td>101.89 ± 16.07</td>
<td>107.70 ± 13.74</td>
<td>105.90 ± 0.93</td>
<td>109.67 ± 17.25</td>
</tr>
<tr>
<td>Blood glucose level at 8 weeks (mg/dl)</td>
<td>100.2 ± 9.59</td>
<td>116 ± 11.05</td>
<td>118 ± 10.91</td>
<td>383.6 ± 17.06</td>
<td>395.67 ± 20.73</td>
</tr>
<tr>
<td>Blood glucose level in terminal samples (mg/dl)</td>
<td>92.6 ± 10.17</td>
<td>169.9 ± 9.8</td>
<td>103 ± 7.3</td>
<td>488.8 ± 17.19</td>
<td>257.56 ± 20.09</td>
</tr>
<tr>
<td>Serum Insulin levels (µIU/ml) (terminal sample)</td>
<td>2.17 ± 0.10</td>
<td>3.11 ± 0.09</td>
<td>3.43 ± 0.14</td>
<td>4.77 ± 0.13</td>
<td>5.07 ± 0.13</td>
</tr>
<tr>
<td>Insulin Resistance HOMA-IR (terminal sample)</td>
<td>0.49 ± 0.05</td>
<td>1.29 ± 0.09</td>
<td>0.87 ± 0.08</td>
<td>5.75 ± 0.22</td>
<td>3.24 ± 0.29</td>
</tr>
</tbody>
</table>

At the end of experiment fasting serum insulin levels were significantly high in group II, IV and V compared with control. There was no significant difference between group III and control. In addition, insulin levels were significantly raised in ghrelin antagonist treated groups compared with their respective untreated groups. The levels were significantly raised in obese group III and diabetic group V compared with obese group II (p = 0.001) and diabetic group IV (p = 0) respectively (Table 2). Insulin resistance (HOMA-IR) was decreased in ghrelin antagonist treated groups compared with untreated groups. HOMA-IR in group III was significantly low compared with group II (p = 0). Group V was significantly low compared with diabetic group IV (p = 0).

Levels of testosterone were increased in ghrelin antagonist treated groups. Although not reaching significant difference, the levels were 34.7% increased in group III compared with group II (p = 0.05). Testosterone levels in group V (72.5% increase) were significantly high (p = 0.006) compared with group IV. The levels of testosterone were significantly lower in all groups compared with the control group I (p < 0.05). The levels of LH were significantly high in group III compared with group II (p = 0) and in group V compared with group IV (p = 0). The levels of luteinizing hormone were significantly lower in all other groups compared with the control group I (p = 0).

Table 3. Serum testosterone and luteinizing hormone levels in BALB/c mice at the end of the experiment

<table>
<thead>
<tr>
<th>Hormonal Profile</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone (pg/ml)</td>
<td>791.11 ± 107.02</td>
<td>434.35 ± 82.80</td>
<td>585.07 ± 124.88</td>
<td>268.19 ± 132.19</td>
<td>462.75 ± 138.75</td>
</tr>
<tr>
<td>Serum LH (mIU/ml)</td>
<td>8.34 ± 0.45</td>
<td>5.16 ± 0.55</td>
<td>7.68 ± 0.24</td>
<td>2.59 ± 0.45</td>
<td>6.34 ± 0.42</td>
</tr>
</tbody>
</table>

Discussion

This study was performed to find the link between obesity, type II diabetes and infertility in males. Previous studies showed that ghrelin, a gastrointestinal hormone, may be involved in this cascade. Ghrelin increases the level of plasma glucose and decreases insulin and testosterone levels. 15, 25 This study was performed to see the reversal of these effects by ghrelin antagonist. We used mice model in our study as has previously been used. 15 High fat diet with 60% fat was given to induce obesity. Diabetes type II and insulin resistance was confirmed in group IV and V by fasting blood glucose levels as previously described.22

Ghrelin is a recently identified potential regulator of both energy homeostasis and reproductive function. Previous studies show that it has a diabetic like effect, increasing blood glucose levels and decreasing plasma insulin levels. 15 It interacts with the reproductive axis, decreasing plasma testosterone levels. 25 On the other hand, Ghrelin antagonist causes a reversal of these effects, causing an increase in plasma insulin levels and normalizing blood glucose. 26 Our results also support the previous studies in animal models where ghrelin antagonist decreased blood glucose levels in normal and obese mice. 15,16,27 This decrease in blood glucose level may be due to an increased secretion of insulin promoted by ghrelin antagonist. Our results showed that insulin levels were significantly increased in both obese and diabetic groups treated with ghrelin antagonist (III and V). The insulin levels were 9.3 % more in obese group III compared with obese group II.
Similarly blood insulin levels were 5.7% more in diabetic group V compared with group IV.  It is suggested that ghrelin antagonist enhances the insulin secretion by blocking ghrelin receptor thus preventing ghrelin inhibitory action on beta cells of pancreas.  

Increase in ghrelin is associated with insulin resistance. Ghrelin depresses the expression and translocation of glucose transporter type 4 (GLUT 4) in muscles causing insulin resistance and ghrelin antagonist leads to its improvement as confirmed by HOMA-IR measurement.  Insulin resistance in antagonist treated groups was significantly low compared with their respective untreated groups (obese group III vs obese group II and diabetic group V vs diabetic group IV). Serum ghrelin levels in obese subjects are positively related to insulin resistance. The significant reduction in HOMA-IR in our study suggests a positive role of ghrelin antagonist in normalizing the plasma glucose levels.

Previous studies suggest a close association between obesity, diabetes, and male reproductive dysfunctions leading to a decrease production of testosterone by the Leydig cells. We also showed that the levels of testosterone in obese group II, was markedly reduced compared with the control. Although we did not measure the enzyme activity in our study, previous studies show that there is an increased activity of enzyme aromatase (involved in conversion of testosterone to estrogen) in the adipose tissue. Enhanced activity of aromatase in obesity causes a feedback inhibition of the gonadotrophin-mediated testosterone secretion. Both these effects lead to a decrease in testosterone production.

Metabolic disturbances like diabetes mellitus also lead to impaired reproductive function by disturbing the pituitary hypothalamic reproductive axis leading to decreased levels of luteinizing hormone, follicular stimulating hormone and testosterone. In rat model, ghrelin significantly decreased the testosterone secretion and produced degenerative changes in the testis by decreasing the stem cell factor, which regulates the development of Leydig cells. Ghrelin has anti-proliferative effects on different testicular cell types and is a negative modulator of male reproductive system, leading to a decreased testosterone production by altering the cellular morphology and affecting different cell organelles. These negative effects of ghrelin on testosterone, luteinizing hormone and follicular stimulating hormone levels are also seen in humans. It is suggested that ghrelin negatively modulates the hypothalamic-pituitary axis and reduces the pulse frequency of gonadotropin-releasing hormone which ultimately suppresses levels of luteinizing hormone. There is a down regulation of Kiss1 expression in hypothalamus that in turn decreases the pulsatile secretion of LH. Few researchers have shown that ghrelin decreases the testosterone synthesis by affecting the enzymes involved in movement of cholesterol from outer to inner mitochondrial membrane or P450cc that is mainly involved in conversion of pregnenolone to testosterone.

Conclusions

1. Glucose level and insulin resistance decrease significantly in obese and type 2 diabetic mice after ghrelin antagonist (D-Lys2) administration
2. Administration of ghrelin antagonist to obese and diabetic groups significantly improves the levels of testosterone and luteinizing hormone

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