Restoration of Libido in WNIN/GR-Ob Male Rats by Administration of High Dose Testosterone Propionate

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Abstract

WNIN/GR-Ob mutant rat strain was isolated and established from our Wistar rat stock and the strain exhibits physical, physiological and biochemical indices of obesity, along with impaired glucose tolerance (IGT). Obese male rats lack libido, have small testis and accessory glands, but with normal spermatogenesis. They are thus infertile, and even testosterone levels were also found to be low. In the present study, we administered testosterone propionate to the animals to see whether this can reverse the reproductive abnormalities and also restore fertility as well. 12 homozygous obese (-/-) male rats of 75-78 days of age were taken and divided into two groups, control and experimental. The experimental animals received a subcutaneous injection of testosterone propionate in thiomersal, and controls received vehicle alone. The experiment was conducted for a period of 90 days. Parameters like growth, food intake and selected biochemical parameters like plasma glucose, insulin, cholesterol, triglycerides and testosterone levels were analyzed in control and experimental animals at the start of the experiment and also before mating with selected proven heterozygous carrier (+/-) females. At the end of the study animals were euthanized and organ weights and body composition were determined. The experimental animals though lost weight, after testosterone propionate injection, gained high testosterone levels, and even successfully mated with the females, which gave viable litters as well. Lipid and insulin parameters all remained high, indicative of clinical obesity. Testosterone administration thus proves to be a method of choice, where one can restore fertility in these animals.

Keywords: Impaired glucose tolerance, Libido, Testosterone propionate, Obese rats.
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Introduction

At our animal facilities, we are maintaining one of the oldest (95 years approx) stocks of rats – Wistar - in an inbred status and hence we refer to this as WNIN, so as to indicate its origin from our Institute, National Institute of Nutrition. From this colony, a mutant line of lean rats showing impaired glucose tolerance (IGT) was earlier identified, showing hypertriglyceridemia, hyperinsulinemia and hypercholesterolemia with a marginal increase in free fatty acids, and was designated as WNIN/GR rats (Giridharan et al., 1997). Around the same time another mutant rat line with obesity and euglycemia was also isolated and a colony of this was also established, and was designated as WNIN/Ob. These mutant rats show high body mass index (BMI), high body fat (47 %) and low lean body mass as compared to lean littermates. They also show hyperphagia, hypercholesterolemia, hypertriglyceridemia, hyperinsulinemia and hyperleptinemia (Giridharan et al., 1996). Subsequently another strain of rats were developed by crossing the WNIN/GR and WNIN/Ob carrier, which combined the traits, i.e., obesity as well as IGT of their parents. The resulting rat strain was called WNIN/GR-Ob as it is obese and exhibits IGT as well (Harishankar et al., 2011 a). This strain of rats have a shorter life span like WNIN/Ob and also develop opportunistic infection as they cross one year, and 15-20 % was shown to develop cataract and retinal degeneration (Reddy et al., 2009), mammary tumors, lipomas and kidney abnormalities (Harishankar et al., 2011 b). In recent times they were shown even to develop hypertension as well (Giridharan et al 2010).

The WNIN/GR-Ob strain is maintained by mating the heterozygous carriers (+/-), which at best could yield 17-20 % of homozygous obese rats over all. This means, that a large number of such mating have to be set apart for obtaining a reasonably good number of homozygous obese animals. There is also an imperative need to identify heterozygous carriers (+/-) at an early age to set up breeding pairs. More than 50% of the animals produced from such mating (carrier Vs carrier) consist of lean and obese, which are of thus no use in propagating the colony and in fact their maintenance amounts to additional expenditure. So, an obvious necessity was felt to reverse the infertility seen in these animals, so that these animals can be put to more efficient use for more production of similar obese animals.

Earlier studies on many such obese rodents showed that, infertility could be reversed by measures that reduce weight gain in obese animals (Dickie et al., 1946), like administration of exogenous testosterone, adrenalectomy and food restriction (Bray et al., 1977; Hemmes et al., 1978; York and Godbole, 1979; Cleary et al., 1980). These measures were found to be highly successful especially with males, since in many of these models,
males exhibit normal spermatogenesis, normal sperm count and motility. They generally seem to be suffering from having inadequate amount of circulating testosterone to induce mating stimuli, and Hemmes and Vaid (1977) showed an even decreased response in male Zucker obese rats to the sex attractant pheromone present in the female urine. In the present mutants, lack of libido appeared to be the limiting factor to successful mating, as the animals have low testosterone levels (Harishankar et al., 2011 c). So, attempts were made in the present study to overcome the infertility problem present in males, by administering testosterone propionate to them.

Materials and Methods

Animals and housing: Twelve homozygous obese males of 77 days age weighing 265 g were taken from our animal facility and were divided equally into two groups - control and experimental. They were housed individually in standard polycarbonate cages with top grill having facilities for holding pelleted feed and drinking water in polycarbonate bottles with stainless steel sipper tubes (Techniplast, Italy). An ambient temperature of 22 ± 2 °C, 14-16 air changes per hour with a relative humidity 50-60% a 12 hour light/dark cycle were maintained. The animals were provided with sterile pelleted chow established at our institute containing all the recommended macro and micronutrients (56% carbohydrate, 18.5% protein, 8% fat, 12% fiber and adequate levels of minerals and vitamins) needed for rats along with water, ad libitum.

Testosterone propionate: The commercially available testosterone propionate (TP) (Aquaviron) manufactured by NICHOlAS, Piramal India limited, Mumbai, was used for the studies. Each ml contained testosterone 25 mg (aqueous adsorbate) in thiomersal I.P. 0.01% W/V. It is a preparation of 4-Androsterone-17β-Propionate-3-one, and CrO₃, with a molecular weight of 344.48. The vehicle thiomersal is sodium ethyl mercury thiosalicylate with a molecular weight of 404.84.

Administration of TP: After initial acclimatization period of three days, the experimental animals at 80 days of age were given a subcutaneous injection of 20 mg testosterone propionate (TP) in thiomersal per rat for the first three consecutive days and 20 mg of TP once in every three days thereafter for a period of 90 days. The control animals received simultaneously, injections of thiomersal (vehicle) alone. Subsequently, each male was mated with two proven heterozygous carrier (+/-) females and the latter were with the experimental males for a period of two estrous cycles. Every day mating cage floor was examined for the presence of vaginal plug as a measure of mounting. After obtaining positive vaginal plugs, another set of two females were introduced, and thus a total of six females were mated with each experimental obese rat during the experiment period. A positive control was also maintained parallely by giving TP to the parental Wistar colony male rats. Similarly females belonging to parental Wistar stock colony were mated with TP treated males at the end of
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The experiment.

**Physical and biochemical parameters:** All the animals in the study were monitored for weekly body weights and also periodically examined for any effect of influence of TP on the body weight from the day of TP administration till 182 days or 26th week– the period when the experiment was terminated. These include physical parameters like growth, body mass index (BMI), food intake. Weekly records were maintained for food intake, weight gain and from these data food efficiency ratios (FER) were derived (Hoover – Plow and Nelson, 1985). Selected biochemical parameters like plasma glucose, insulin, cholesterol, triglycerides and plasma testosterone levels were also measured at the start of the experiment and at the time of euthanization of animals. For oral glucose tolerance test (OGTT), an oral glucose dose of 250 mg/100 g body weight was given through a gavage and the blood was drawn. To carry out biochemical parameters, the animals were fasted for 17 hours and the initial blood sample was collected under mild anesthesia (Isoflurene) with a heparinized capillary tube from the supra-orbital sinus of the eye via the inner canthus by using heparinized micro capillary tubes as illustrated by Riley (1960). The blood was collected in test tubes containing 20 mg of sodium fluoride as anti-coagulant, plasma was separated from the cells and stored at -20°C for analysis.

**Organ weights and Body composition:** At the end of the experiment (i.e., 26th week), rats were fasted for 17 hours and euthanized by CO₂ inhalation and subjected to gross necropsy. External features suggesting any abnormality were looked into. After opening the viscera an **in situ** examination was done. The major organs like liver, heart, lungs, kidneys and testis were collected. The individual organs were examined for gross morphology changes after removal. After detailed gross necropsy examination, they were trimmed of fat and blotted on filter paper and weighed (Bindhu *et al.*, 2007) (Essae Digi analytical balance, ES-DIGI, India). After noting down the body weights the organs were placed in the carcass for body composition analysis by chemical method, which was carried out as per the modified procedure of Rathburn and Nellopace (1945).

**Statistical analysis:** Statistical significance of differences between the groups was determined by student’s unpaired “t” test. Two way ANOVA was carried out using Duncan’s multiple range test, when multiple comparisons were involved. Where ever the data was found to be highly variable, the log transformation was carried out and further tested with repeated measures of ANOVA. A *p* value of 0.05 was considered statistically significant.

The study was reviewed and approved by the Institutional Animal Ethical Committee (IAEC), and was conducted in accordance with the internationally accepted principles for laboratory animal use and care.

**Results:** Weekly body weights and food intake are given in Fig.1 and Table 1. There was a significant reduction in food intake in testosterone treated animals along with body weights compared to thiomersal injected rats (*P*<0.001). FER values were significantly different in both TP treated and thiomersal injected rats (*P*<0.001). At the end of experiment, there was
a significant reduction (P<0.001) in the body weight of experimental animal group, as compared to thiomersol injected rats. Changes in body weight with time are shown in Fig.1. The rate of weight gain was significantly reduced as early as 30 days after TP treatment had begun. This resulted in a significant (P<0.05) reduction in the body weight and BMI of TP treated rat at the end of experiment.

The treated animals continued to have impaired glucose tolerance however with a significant reduction (P<0.001) in the plasma insulin levels compared to control (Table.2). There was no significant change in the levels of plasma cholesterol in experimental and control rats. However, a significant increase in triglyceride and circulating testosterone levels were noted in TP treated rats compared to its control rats (Table.2).(P<0.01)

Significant differences were seen in the total weight of major organs as well as the ratio of body weight to organ in TP treated rats as compared to controls as per the method described by Michael, B (2007). Significant decrease in the weight as well as body weight to organ ratio was seen in liver, lungs and kidneys of TP treated rats. The testis weight in TP treated animals is significantly increased as compared to thiomersol treated group. There was a significant reduction in the body weight as well as total body fat with a concomitant increase in the lean body mass in the TP treated obese rats as compared to control rats. Body composition of TP treated rats is given in Table 3. Body composition of both experiment and control obese rats was carried out by carcass chemical method and the results were given in Table. 4. There was a significant reduction (P<0.05) in the body fat content in TP treated rats compared to thiomersal injected rats, with a concomitant increase in lean body mass in the former. The percent of total body fat, fat free mass and body water also significantly reduced in TP treated rats as compared to thiomersal treated rats.

100 % of conception was seen in the females mated with the TP treated males and an average litter size of 8.40 ±1.10 per rat was seen, while the control animals failed to do so. The progeny contained 45.65 % male and 52.50 % female heterozygous carriers and 54.35 % male and 47.50 % female homozygous obese rats. The positive control, WNIN rats treated with TP lost weight considerably, and they also failed to impregnate the females.

Discussion

It was shown earlier, that testosterone treatment does decrease food intake and reduces body weight in Zucker obese males. This was attributed to a beneficial effect of testosterone administration. In these studies massive dose of testosterone injection improved fertility in young obese male Zucker rats (3-4 months old) with no effect in 7-11 months old ones9. Similar observations were made by others as well and this seems to be a sure way of restoring the fertility in such mutants. We tested the same possibility in our infertile WNIN/GR-Ob rats, at an age close to the mating age i.e., 90 days.

Earlier, we have seen that these obese male rats have low gonadal and accessory
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Gland weights, low or no libido, with low levels of circulating testosterone (Harishankar et al., 2011c). All the TP treated obese male’s successfully impregnated females which produced viable litters as well. As mentioned, this was earlier tried with Zucker rats as early as 1976 (Hemmes et al., 1978), and subsequently followed by many commercial groups to produce Zucker obese rats. However, the decrease in testosterone levels in those animals was not as substantial as seen in our obese mutants. It should be remembered that 20 mg of TP is really a very high dose, and in fact this is a contraceptive dose for a normal rat, as it was observed here with parental normal Wistar rats, which were used as control. However, in obese conditions, such dosage of TP seem to cause reduction in food intake and FER, as well as 20-25% weight reduction as shown earlier by Hemmes et al., (1978). But it should be noted that though these animals recovered their libido and also became trimmer, the cholesterol and triglycerides levels did not change much and however, on the contrary the latter was on the rise as well. Same case with plasma insulin, which showed a 50% reduction, is however still above the normal levels. The IGT nature of the mutant also did not change. This means that, though TP treatment could reverse their infertility (as a result of lack of mounting behaviour) the obesity per se was not altered. This was supported by BMI and carcass analysis of these rats as well. As suggested by Glass et al., (1977), aromatization of TP by adipose depots may be the prime cause for testosterone deficiency seen in the present mutants. In the present study, on testosterone treatment, the body fat in obese rats came down to 37% compared to 47% fat in control. This may or may not be important for the successful mating of the mutants, since excess testosterone is already been provided by way of injections. Hypophagia and reduction in insulin levels might be additional benefits, though not the direct cause for reversal of infertility.

Fertility restoration in other obese rodent models was described as a tough task, and at times, food restriction as well as TP injection was given in combination to obtain the desired results (Ruth Kawa et al., 1990). Reports on such attempts are sparse in literature and not many papers are published on this aspect. Our study is one of the few studies on fertility restoration with such detailed biochemical analysis. Based on the data presented here, this stock of WNIN obese mutants appear to be more amenable to exogenous supplementation of TP in restoring fertility. It should be remembered that initially the colony could be maintained only by mating heterozygous carriers (+/−), resulting in lesser percentage of obese animals and higher production cost. But now with homozygous obese male animals (−/−) per se being available for successful mating, the production of both carrier as well as obese can go up substantially (as shown our mating data), with lesser cost in put. In other words, from a mere 17-20% of production of homozygous obese rats by carrier X carrier mating, the production can now be stepped up to 50% and above, by restoring fertility to these homozygous obese male rats.

In short, the experiment reported here, showed that administration of TP is an effective procedure for the successful production of homozygous obese animals in large numbers, without losing any of the biochemical characteristics of obesity. The
administration of TP resulted in the body weight reduction, increased androgen production, leading to successful mating and large litter production, consisting of equal number of heterozygous carrier (+/-) and homozygous obese (-/-) animals.

Acknowledgements

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**Figure 1:** Growth pattern of control and experimental obese rats. Each line represents the mean of observations in 6 rats of each group. Means with *** are significantly different at $p<0.0001$, by two-way ANOVA followed by post hoc least significance test.
Table 1: Body mass Index (BMI), body weight, food intake and FER of TP and thiomersal treated rats. (n=6), during the experimental period.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMI</th>
<th>Weight at 70 days</th>
<th>Weight at 182 days</th>
<th>Weight gain</th>
<th>Food Intake</th>
<th>FER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiomersal (Control)</td>
<td>5.34 ± 0.18</td>
<td>8.34 ± 0.40</td>
<td>263.00 ± 9.74</td>
<td>566.00 ± 32.06</td>
<td>303.00 ± 17.49</td>
<td>133.40 ± 18.42</td>
</tr>
<tr>
<td>TP treated (Experimental)</td>
<td>5.34 ± 0.13</td>
<td>7.35 * ± 0.48</td>
<td>265.33 ± 6.91</td>
<td>444.33 *** ± 12.38</td>
<td>179.00 *** ± 11.89</td>
<td>102.25 *** ± 13.31</td>
</tr>
</tbody>
</table>

TP – Testosterone propionate. BMI – Body mass index. FER – Feed efficiency ratio. Values are mean ± S.E. * P < 0.05, *** P < 0.001. Significant by Duncan’s Multiple range test.
Table 2. Selected biochemical parameters in TP and thiomersal treated rats. (n=6)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose(AUC) (mg/dl)</th>
<th>Insulin (U/mol)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiomersal (Control)</td>
<td>298.88 ± 13.92</td>
<td>402.28 ± 15.54</td>
<td>70.24 ± 7.65</td>
<td>225.15 ± 7.35</td>
<td>3.08 ± 0.98</td>
</tr>
<tr>
<td>TP treated (Experimental)</td>
<td>294.85 ± 6.92</td>
<td>264.95 *** ± 6.50</td>
<td>81.55 * ± 2.43</td>
<td>250.69 *** ± 15.58</td>
<td>23.02 *** ± 2.38</td>
</tr>
</tbody>
</table>

Table 2. Selected biochemical parameters in TP and thiomersal treated rats. (n=6)

TP – Testosterone propionate. AUC – Area under the curve.
Values are mean ± S.E. * P < 0.05, *** P < 0.001. Significant by Duncan’s Multiple range test.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Organ weights</th>
<th>Organ to body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Heart</td>
</tr>
<tr>
<td>Thiomersal (Control)</td>
<td>13.25</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>± 0.49</td>
<td>± 0.04</td>
</tr>
<tr>
<td>TP treated (Experimental)</td>
<td>9.70*</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>± 0.55</td>
<td>± 0.06</td>
</tr>
</tbody>
</table>

Table 3. Organ weights and organ to body weight ratio in TP and thiomersal treated rats. (n=6).

TP – Testosterone propionate.
Values are mean ± S.E. *P < 0.05. Significant by Duncan’s Multiple range test.
### Table 4. Body composition by chemical method in TP and thiomersal treated rats. (n=6).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (g)</th>
<th>LBM (g)</th>
<th>Body fat (g)</th>
<th>Body fat %</th>
<th>FFM (g)</th>
<th>TBW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiomersal (Control)</td>
<td>566.00 ± 32.06</td>
<td>254.00 ± 4.86</td>
<td>312.00 ± 8.17</td>
<td>55.12 ± 2.08</td>
<td>133.40 ± 18.42</td>
<td>25.28 ± 1.10</td>
</tr>
<tr>
<td>TP treated (Experimental)</td>
<td>444.33 *** ± 12.38</td>
<td>292.33 ± 6.33</td>
<td>152.00 *** ± 12.08</td>
<td>34.21 * ± 3.68</td>
<td>102.25 *** ± 13.31</td>
<td>19.38 *** ± 1.17</td>
</tr>
</tbody>
</table>

TP – Testosterone propionate. LBM – Lean body mass; FFM – Fat free mass; TBW – Total body water.

Values are mean ± S.E.  * P < 0.05, *** P < 0.001. Significant by Duncan’s Multiple range test.

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