Effects of aproteic diet on hypothalamic-pituitary-gonadal regulation in the male rat

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Abstract: The effect of an aproteic diet (Ap) on the reproductive axis in young male rats was studied. Also the refeeding effect at different times after the aproteic diet was studied.

The Ap diet was given during 21 days. In refeeding groups, the control diet was given during 2, 4 and 6 weeks after the aproteic diet. We studied the plasmatic testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels. Also the hypothalamic GnRH concentration and in vitro hypothalamic GnRH secretion in basal and induced condition was studied.

The total protein deficit produced significant reduction in body, testis, seminal vesicles and prostate weights. This was accompanied with decreased levels of plasmatic testosterone (P<0.02). In this aproteic group there was a significant reduction in LH (P<0.05) and FSH (P<0.05) plasmatic levels. Refeeding with control diet reversed this situation, producing significant increment in LH (P<0.05) and FSH levels (P<0.01) at the fourth and second weeks, respectively. The basal hypothalamic GnRH secretion did not differ from the control; nevertheless the induced secretion was significantly (P<0.05) greater in the aproteic group. Also the hypothalamic GnRH concentration was increased (P<0.05) in animals fed with the aproteic diet.

The minor testis, prostate, and seminal vesicles’ weight, and a decreased plasmatic testosterone in rats fed with an aproteic diet, are produced by a decrease in gonadotrophin secretion. This decrease in turn is caused by a reduction in GnRH secretion, since hypothalamic GnRH concentration is increased in rats fed with the aproteic group, and induced secretion is greater in this group. All these alterations produced by an aproteic diet are reversible, since—with control diet refeeding—the gonadotrophin secretion returned at control levels.
Introduction

Changes in the nutritional status influence the activity of the reproductive system in many species. A fasting situation, in which the animal suffered the deficit not only of the different macronutrients necessary for normal development but also of calories (Warnhoff et al. 1983; Trimino et al. 1991; Cameron and Nosbisch 1991), can induce an abnormal reproductive situation. Vaginal opening and pubertal development is delayed and associated with alteration on plasmatic gonadotrophin levels in female rats; gonadal atrophy and infertility are some reproductive alterations in adult male rats and men (Zubieta 1981; Zubieta et al. 1983). This same alteration was observed in women with secondary amenorrhea that are gymnasts or distance runners and have caloric restriction, the same alteration (Williams et al. 1985) can be observed. All this work does not take into account the macronutrient deficit or the calorie deficit.

To conduct this work, we avoided the deficit of macronutrients like carbohydrates, fats, minerals and vitamins—also the fundamental caloric restriction that other works do not take into account. In this model we studied the alteration of the reproductive axis caused by a diet that is deficient in protein and isocaloric with a control diet.

Materials and Methods

Animals and experimental design: the experiments were performed using young adult male Wistar rats taken from the Department of Physiology, School of Medicine, University of Buenos Aires. They were housed in an air-conditioned (22–24°C), light-controlled (lights on from 07:00–19:00 hours) room. Until the experiment began, the rats were fed with a balanced commercial ration. Fifty 60-day-old rats with 230 to 260 g body weight were put in each cage.

All the animals were divided into two groups: half received a control diet with enough quantity of protein (20%), caloric and other necessary macronutrients, while the rest (the other half) was fed with a free protein diet, isocaloric with a control diet. The two groups received water ad libitum. This treatment was extended for 21 days. Body weight was monitored every week as a parameter of the animals’ state. At the conclusion of this period, the animals’ were decapitated within 12 and 13 hours immediately after being removed from the cage. Some animals from the aproteic group were fed with a control diet during 2, 4 or 6 weeks, and then sacrificed. Some animals were also fasting for 7 days in order to establish the difference with an aproteic diet.

Processing of the sample: trunk blood was collected immediately after decapitation. The serum obtained was stored at -80°C until hormonal assay by RIA, LH and FSH were measured in aproteic, refeeding and fasting groups, and testosterone was measured in the aproteic group. Seminal vesicles, testis and prostate glands were also dissected in the aproteic group animals in order to weigh them. Brains were rapidly removed, and the hypotalami dissected with a single razor blade. The hypothalamic samples containing the medial basal hypotalami and the anterior and preoptic hypothalamic areas were dissected with the help of a stereomicroscope. The hypothalamic samples were border laterally by the hypothalamic sulci, rostrally 3 mm anterior to the optic chiasm, and caudally by the mamillary bodies, the depth was 3-4 mm. Half of them were frozen (-80°C) to determine GnRH tissue concentration. The other half was incubated using a fresh medium to study the hypothalamic GnRH secretion.

Hypothalamic incubation: immediately after being dissected, the anterior mediobasal hypotalami were put into plastic chambers containing 500 ul of Earle’s medium with glucose (1 mg/ml) and Bacitracin (20 mM) to prevent GnRH degradation (Rotsztejn et al. 1976). The pH value was adjusted to 7.4. Each chamber with hypothalamic tissue was incubated in a Dubnoff shaker at 37°C with constant shaking (60 cycles/min) under an atmosphere 95% O₂ / 5% CO₂. After 30 minutes of preincubation, the medium was changed by adding fresh Earle’s medium, containing CIK 5.6 mM. After 15 minutes of incubation the medium was collected (basal samples) and immediately frozen at -80°C. The tissue was washed twice with the same medium and then incubated one more time with Earle’s medium 56 mM (induced sample). The samples were taken in 10 ul chloridric 0.1 N and immediately frozen at -80°C until RIA assay.

Hypothalamic homogenization: anterior-mediobasal hypotalami were weighted to 0.01 mg, and then homogenized in 1 ml chloridric 1 N, using an all glass Potter homogenizer refrigerated with ice. The homogenate was centrifuged at 13000 rpm during 2 minutes, and the supernatant obtained was diluted 1:25, and then frozen at -80°C until GnRH assay by RIA. The results were expressed in nanograms per mg of tissue.

Hormone assay: plasmatic LH and FSH levels were determined using a double antibody RIA tech-
tique (Niswender GD et al. 1968). The material for these assays was kindly provided by the NIAMDD rat pituitary programme (Bethesda, MD). GnRH levels were measured by the RIA method described elsewhere (Clough 1988), after neutralization of the samples with 0.5 N NaOH (10μl). GnRH antiserum (final concentration 1:30,000) was kindly provided by Dr. V.D. Ramirez; [1 125] GnRH was purchased from New England Nuclear, Boston, MA., USA; synthetic GnRH (Peninsula Laboratories, Belmont, CA, USA) served as reference. The sensitivity of the assay was 0.2 pg/100μl; intra- and interassay variation coefficients were 7% and 14%, respectively.

Statistical analysis: The results are expressed as mean ± SEM. For organ weight, testosterone and GnRH concentration, statistical differences were tested using Student test “t.” For gonadotrophin differences, ANOVA was followed by Bonferroni’s test (Bonferroni Multiple t Test. Instat Programme GraphPAD. San Diego, CA, USA). For GnRH secretion Student “t” test for paired samples was used. A P level <0.05 was considered significant.

Results

After 21 days of an aproteic diet, the rats suffered a fall of 32% in body weight (P<0.001), with respect to their initial weight. There was a difference of 40% in the final body weight with respect to the controls animals. After 7 days of fasting, the rats’ body weight also descended significantly (P<0.001), being 24% less than the controls. The refeeding after an aproteic diet increased the body weight, and after six weeks there was no significant difference with the controls rats (body weight 94% of control weight).

The aproteic diet lowered significantly the testis (P<0.01), seminal vesicles (P<0.001) and prostate gland (P<0.001) weight (Table 1). In the male rats fed with an aproteic diet, the minor weight of the testis was accompanied by a significant (P<0.02) decrease in testosterone plasmaic levels, reaching the 50% levels of the controls (Figure 1).

Table 1. Effect of an aproteic diet during 21 days on organ weight (mg of fresh tissue). Results expressed as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control Diet</th>
<th>Aproteic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td>1693 ± 37</td>
<td>1535 ± 30</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>498 ± 20</td>
<td>165 ± 14</td>
</tr>
<tr>
<td>Prostate gland</td>
<td>435 ± 30</td>
<td>206 ± 20</td>
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</tbody>
</table>

In animals fed with an aproteic diet, the plasmatic concentration of LH (P<0.05) and FSH (P<0.05) was significantly decreased, with respect to the control animals. When the animals were refed with the control diet, the plasmatic LH concentration was significantly incremented over aproteic (P<0.001), and even over control levels (P<0.05) in 4 weeks of refeeding. In six weeks it returned to control levels (Figure 2). The FSH was significantly (P<0.01) incremented in two weeks of refeeding, reaching control levels (Figure 3). The LH (P<0.05) and FSH (P<0.01) also decreased significantly in fasting rats.

The incubation of mediobasal hypothalamus showed a different answer to the CLK 56 mM in aproteic animals respecting control rats (Figure 4). Leaving similar levels of GnRH basal hypothalamic...
secretion in the aproteic and control groups, the depolarization with CLK 56 mM stimulated significantly the secretion both in the control (P<0.05) and aproteic (P<0.01) groups. This increment on the GnRH liberation was significantly major (P<0.05) in animals fed with an aproteic diet with respect to controls animals. The percentage of increment with respect to basal levels, in aproteic group (214.9 ± 29.2%), was also significantly (P<0.05) greater than in the control (115.6 ± 33.9%) group. The hypothalamic GnRH contents were significantly (P<0.05) incremented in rats fed with the aproteic diet (Figure 5).

**Discussion**

The results obtained in this experience clearly indicate that the sole fault of protein in the diet during 21 days has some influence on the control of the reproduction system. These results are similar to those obtained in a complete fasting or partial restriction situation. Like a fasting situation, an aproteic but normocaloric diet, plus sufficient amount of minerals and vitamins, produces a decrease on plasmatic gonadotrophin levels in young male Wistar rats that lost 32% of their body weight. In this case the exclusive deficiency of protein is able to produce the dysfunction in the reproductive axis function.

This model of the aproteic diet is able to produce a decrease in LH and FSH pituitary secretions in adult Wistar rats. It has been shown that a faulty protein diet can produce a decrease in basal LH plasmatic levels in growing female sheep; accompanied with this is a delay in the puberty development (Olkowska and Prezekap 1993). Other experiences using fasting as an experimental model have demonstrated not only the LH plasmatic levels decrease, but also the decline of plasmatic testosterone levels (Srebnik 1970; Root and Russ 1972; Howland and Skinner 1973; Campbell et al. 1977). In this work we demonstrated that the specific fault of protein is also able to produce a decrease/reduction in plasmatic testosterone levels. This is so, although Leydig cells could be able to produce testosterone in a fasting situation, since the stimulation with hCG in these rats produces an increase in testosterone secretion (Pirke and Spyra 1981; Bergendahl et al. 1989).

Likewise the desnutrition is able to block the important LH rise that occurs after the castration of male rats if it is imposed immediately after the surgery, while the FSH response does not change (Root and Russ 1972) nor an alteration in gonadotrophin
pituitary synthesis (Root and Russ 1972; Howland and Skinner 1973). All these results are consistent with a report of decreased plasmatic gonadotrophin during one anorexia nervosa in a subject with gonadal disgenesism (Kauli et al. 1982). To study the hypothesis that the cause of gonadotrophin secretion alteration is not in hypophysis, but in upper levels like the hypothalamus, we measured the GnRH concentration in the hypothalamic tissue, which is known to be a stimulus to provoke gonadotrophin secretion. We found that anteromedialbasal included the medial eminence, the hypothalamic concentration of GnRH was significantly increased in animals fed with an aprotic diet, respectively rats fed with a normoproteic diet. The results agree with those reached by Totman, who demonstrated the cessation of LH release with an elevated quantity of GnRH in medial eminence of castrated ewes with energetic deficiency, probably as the result of a decrease in its secretion (Totman et al. 1990). The immunoreactive content of GnRH stored in the median eminence was enhanced in growing male lambs with protein deficiency (Polkowska 1989).

Some other works did not find significant differences when studying the content in the preoptic area, and found a slight increase in the hypothalami of fasting animals; although when they isolated the medial eminence, they found a significant increase in GnRH concentration that was twice as high as in the starved rats (Pirke and Sprya 1981).

The suppression of LH and FSH liberation in adults could be generated by a damage in hypothalamic GnRH secretion. This hypothesis agrees with our results, because we observed a significant increment in GnRH secretion by the hypothalamus after a great depolarization of tissue by CLK 56 mM with respect to the controls. These results are talking about a blocker on GnRH secretion in the live animals, because the tissue concentration was increased and basal liberation was similar in both groups. It is interesting to note that the pituitary levels of the GnRH receptors are decreased about 50% after 4 to 6 days of food deprivation (Bergendahl et al. 1989).

The intermittent administration of GnRH to castrated adult male Rhesus monkeys, in which the gonadotrophin secretion was decreased after a restriction of diet, was able to restore completely the circulating LH and FSH at levels seen before the diet (Foster and Olster 1985; Dubey et al. 1986; Bronson 1986). In the same way, patients with anorexia nervosa treated with GnRH or some of its analogue, renewed the menstrual ciclicity (Nillius et al. 1975; Zgliczynski et al. 1984). Furthermore, an inadequate protein consumption could affect some excitatory or inhibitory aminoacid way (Barraclough and Wise 1982), because the administration of aminoacid stimulates the secretion of LH in desnutrition ewes (Bucholtz et al. 1988). Moreover it had been demonstrated that the excitatory aminoacid system plays an important role in the regulation of gonadotrophin secretion, acting at hypothalamic levels (Gay and Plant 1987; Bourguignon et al. 1989; Brann and Mahesh 1992).

In conclusion an absolutely free protein, but isocaloric with a control diet, is able to produce alteration of the gonadal axis function, manifested by a decrease in the weight of sexual organs, plasmatic testosterone, and gonadotrophin levels. This is produced by a damage in hypothalamic GnRH liberation. This event is related to an increment in hypothalamic GnRH concentration in animals that received the aprotic diet. The alterations would not be definitive, because refeeding with a control diet can restore the gonadotropic secretion. If the cause of the GnRH damage is the consequence of alterations in higher regulatory levels, this will be studied in future works.

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