Effect of casein diet on gonadotropin releasing hormone antagonist induced changes in adrenal gonadal functions in male rats

N M Biswas, Gargi Ray Chaudhuri, A Chattopadhyay & M Sarkar
Reproductive Physiology Unit, Department of Physiology,
University College of Science and Technology, 92, A.P.C. Road, Calcutta 700 009, India

Received 12 April 2001; revised 17 August 2001

Adult male rats received daily injections (sc) of gonadotropin releasing hormone antagonist (0.2 mg/kg day) for 21 days when they were sacrificed on day 22, adrenal weight, adrenal Δ2-3β (delta 5-3beta) hydroxysteroid dehydrogenase (Δ2-3β-HSD) activity and serum level of corticosterone were increased significantly while testicular 17β (17beta) hydroxysteroid dehydrogenase (17β-HSD) activity and serum level of testosterone and spermatogenesis were decreased in the rats fed on 5% casein diet. GnRH antagonist treated rats fed on 20% casein diet, resulted significant decrease in adrenal weight, serum corticosterone and adrenal Δ2-3β-HSD activity while testicular 17β-HSD activity serum testosterone levels and the weights of sex organs were increased with respect to anti GnRH treated rats fed on 5% casein diet. But the GnRH antagonist treated rats fed on 20% casein diet showed decreased spermatogenesis quantitatively and sperm count appeared similar to anti GnRH treated rats fed on 5% casein diet. These results indicate that high casein diet protects adrenocortical activity and stimulates testosterone synthesis without effecting spermatogenic arrest in GnRH antagonist treated rats. It may be concluded that GnRH antagonist in presence of high milk protein diet may be considered to be a suitable antihormone in the development of an ideal male contraceptive.

Gonadotropin releasing hormone antagonist affects spermatogenesis by inhibiting gonadotrophins release and subsequently testosterone production\(^\text{1,2}\). Although attempts have been made to use GnRH antagonists as male contraceptives but unaccepted side effects and suppression of testosterone made them impossible to use in human practice. Side effects due to GnRH antagonists include oedema of the face and extremities accompanying release of histamine\(^\text{3}\). Since hypothalamic histamine stimulates corticotropin release by activating corticotropin releasing hormone-containing neurons\(^\text{4}\), there is a possibility (still unproved) that GnRH antagonist treatment may cause adrenal hyperactivity due to excess corticotropin release.

In a previous paper we have demonstrated that consumption of casein enriched diet prevents adrenal hyperactivity in rats\(^\text{5}\) and the suppression of testicular androgen synthesis without effecting spermatogenic inhibition (unpublished results of N.M.B.) in estrogen-treated rats. Therefore, we have investigated the effect of casein diet on adrenal and gonadal activities in GnRH antagonist treated rats.

Materials and Methods

Animals and GnRH antagonist treatment—Adult male Wistar rats weighing 150-160g (120-140 days of age) were used in this experiment. 18 rats were housed in a laboratory controlled environment at 30°C with 14 hr illumination daily. All the rats were fed ad libitum on two series of diets described previously\(^\text{6}\). First series of diets contained 5 g casein (P.C. Dutta and Bros, Calcutta, India), 38.5 g wheat meal and 46.5 g chick-pea; second series 20 g casein, 39 g wheat meal, 31 g chick-peak in addition to the corn oil 5 g, vitamin mixture 1 g, salt mixture 4 g in 100 g of both the diets. The total protein content of both the diets was about 15%.

The animals were divided equally into 3 groups. Two groups of rats received diet containing 5% casein and other group 20% casein. One group of 5% and 20% casein diet fed rats were injected subcutaneously with GnRH antagonist (D-Phe\(^\text{5}\), D-Ala\(^\text{6}\)-LHRH) Sigma Chemical Co.; St. Louis, Missouri, U.S.A.) for 21 days (0.2 mg/kg day\(^{-1}\)) dissolved in 0.2 ml vehicle composed of distilled water : propylene glycol (1:1). The remaining 6 control rats fed on 5% casein diet received vehicle only. One day after the last injection of GnRH antagonist, all the animals were killed by decapitation under light ether anesthesia following protocols and ethical procedure. Blood obtained from all the animals was centrifuged for serum separation. The testis, epididymis, seminal vesicle, ventral prostate and adrenal were dissected
out and weighed. The right testis of each rat was fixed in Bouin's fluid for histological study. The Adrenal glands and left testis of each rat were kept at -20°C and then immediately processed for enzyme analysis as described below:

### Measurement of adrenal and testicular enzyme—Adrenal and testicular $\Delta^2$-3$\beta$-HSD and testicular 17$\beta$-HSD activities were assayed following the methods of Talalay and Jarabak respectively. Testicular and adrenal tissue homogenised separately with homogenising fluid containing 20% spectroscopic grade glycerol, 5 mM potassium phosphate and 1 mM EDTA at a tissue concentration 25 mg ml$^{-1}$ homogenising mixture and centrifuged at 10,000 g for 30 min in an ultracentrifuge at constant temperature of 4°C. For studying the activity of $\Delta^2$-3$\beta$-HSD 1 ml of supernatant of adrenal/testicular homogenising fluid was mixed with 100 $\mu$mol sodium pyrophosphate buffer (pH 8.9), 0.9 ml double distilled water and 30 $\mu$g DHEA making incubation mixture a total of 3 ml. Enzyme activity was measured after the addition of 0.5 $\mu$mol of NAD to the tissue supernatant mixture in an UV spectrophotometer (Beckman) at 340 nm UV against a blank without NAD. One unit of enzyme activity was equivalent to a change in absorbancy of 0.001 min$^{-1}$ at 340 nm. For 17$\beta$-HSD activity 1 ml of the same testicular supernatant was mixed with 440 $\mu$mol sodium pyrophosphate buffer (pH 10.2), 25 mg crystalline bovine serum albumin and 0.3 $\mu$mol testosterone making the incubation mixture a total of 3 ml. Enzyme activity was measured after the addition of 1.1 $\mu$mol NADP to the supernatant mixture in a spectrophotometer at 340 nm against a blank without NADP. One unit of enzyme activity was equivalent to a change in absorbancy of 0.001 min$^{-1}$ at 340 nm.

### Study of spermatogenesis—Paraffin-wax sections (5 $\mu$m thick) were taken from the mid portion of each testis and stained with periodic-acid Schiff haematoxylin. The relative number of each variety of germ cell at stage VII of the seminiferous epithelium cycle, i.e. type-A spermatogonia (ASg), preleptotene spermatocytes (pLSc), mid-pachytene spermatocytes (mPSc) and step-7 spermatids (7Sd) were counted according to the method of Leblond and Clermont. The different nuclei of germ cells except for step-19 spermatids which cannot be enumerated precisely) were counted in 20-22 round tubular cross-sections at stage VII in each rat. All of the nuclear counts of the germ cells were corrected for differences in nuclear diameter by the formula of Abercrombie, i.e. true count=(crude count x section thickness) / (section thickness + nuclear diameter of germ cells) and tubular shrinkage by Sertoli cell correction factor.

### Sperm count—The sperm count was determined by haemocytometer. The cauda epididymis was punctured and placed in a beaker containing 5 ml 1% (w/v) fructose in phosphate buffer (pH 7.4). A drop of suspension was placed on the haemocytometer. The number of spermatozoa in five small squares were recorded. To minimize the error, each count was repeated at least five times for each testis.

### Measurement of serum corticosterone and testosterone—Serum corticosterone was determined by spectrofluorometry according to the methods of Glick and Silber. The fluorescence was measured at 463 nm (excitation) and 518 nm (emission) by setting the instrument at a spectrofluorometric reading 80 with a standard corticosterone (Sigma Chemical Company, St. Louis, MO, USA) solution having the concentration 1.6 $\mu$g/ml. A minimum 1.6 $\mu$g of corticosterone/100 ml can be measured by this method.

Radioimmunoassay of testosterone was carried out using testosterone $^{125}$ I RIA Kit (ICN Biochemical Inc., Diagnostic Division, Costa Mesa, CA 92626, USA). Radioactivity was determined by the Gamma Scintillation Counter at Bose Institute, Calcutta, India. All samples were run in duplicate in a single assay to avoid interassay variations. The interassay coefficient of variations for testosterone was 6.5%. Since chromatographic purification of the sample was not performed, each testosterone value was the sum of testosterone and dihydrotestosterone.

### Results

There was a significant increase in adrenal weight, adrenal $\Delta^2$-3$\beta$-HSD activity and serum level of corticosterone in rats fed on 5% casein diet and treated with GnRH antagonist in comparison with controls (Table I & Fig.1a&b). But GnRH antagonist treated rats fed on 20% casein diet did not show significant increase in adrenal weight and $\Delta^2$-3$\beta$-HSD activity although serum level of corticosterone (Fig.1,b) was higher than the control values but lower than 5% casein fed group treated with GnRH antagonist. Treatment with GnRH antagonist resulted in a significant decrease in the weights of the testis, seminal vesicle and ventral prostate after 21 days in comparison with control animals. No change in body weight was observed in
BISWAS et al.: EFFECT OF CASEIN DIET ON ADRENAL & GONADAL ACTIVITIES

GnRH antagonist treated rats fed on 5% or 20% casein diet (Table 1). Testicular 17β-HSD activity and serum level of testosterone were decreased in GnRH antagonist treated rats fed on 5% casein diet. After feeding 20% casein diet testicular 17β-HSD activity, serum levels of testosterone and the weights of sex organs were increased in GnRH antagonist treated rats compared to 5% casein fed rats treated with anti GnRH but showed lower than the controls. The immature spermatozoa in the lumen of seminiferous tubules at stage VII and epididymal mature spermatozoa count (Table 2; Fig.1d) were lower in both 5% and 20% casein fed rats treated with GnRH antagonist when compared with controls. The quantitative study of germ cells at stage VII (Table 2) showed reduction in relative number of pLSc, mPSc and 7Sd in antagonist treated rats fed on 5% and 20% casein diet in comparison with 5% casein fed controls.

Discussion

This study has demonstrated that high casein diet prevents adrenocortical hyperactivity and suppression of serum testosterone levels without altering spermatogenic arrest in GnRH antagonist treated rats. Whether this stimulating effect of the antagonist on the adrenals is direct or through the increased release of hypothalamic corticotropin releasing hormone (CRH) is yet to be determined.

Gonadotropin releasing hormone antagonist induces release of hypothalamic histamine which has the capacity to stimulate corticotropin release by activating CRH containing neurons. So the increased adrenal Δ4-3β-HSD activity and serum corticosterone level in anti GnRH treated rats fed on 5% casein diet may be due to excess release of ACTH by stimulated corticotropin secretion. There are other studies, however, which suggest that normally endogenous GnRH affects the functions of several extra hypothalamic tissues through its receptors. Since the binding sites for GnRH appear to be present on adrenal gland, possibility remains that anti-GnRH may stimulate adrenal function after blocking the inhibitory effect of endogenous GnRH on the adrenal.

Several studies in male rats have shown that cortisol inhibits the release of LH after orchidectomy. Inhibitory effects of glucocorticoids on LH secretion have been reported in cultured pituitary. Glucocorticoids also directly suppress testosterone production and secretion by decreasing testicular LH receptors. Treatment of Leydig cell culture with a glucocorticoid hormone results in decreased level of cholesterol side-chain cleavage enzyme P450sc. On the other hand cortisol has been found to suppress LH secretion and combined treatment of GnRH antagonist and cortisols results in further suppression of serum LH but has no effect on serum FSH. So greater reduction of LH than FSH secretion observed in anti GnRH treated primates is possibly due to increase in cortisol secretion.

In the present experiments rats fed on casein enriched (20%) diet show a significant increase in serum testosterone in anti GnRH treated group as compared to 5% casein fed treated rats. Therefore, the increased serum levels of testosterone and testicular 17β-HSD activity in anti-GnRH treated rats fed on...
20% casein diet indicates that the secretion of pituitary LH or the testicular LH receptors may have been stimulated. There are other studies, however, that shows adrenocortical hyperactivity and fall of serum levels of FSH and LH in estrogen-treated rats but feeding of high casein diet increases serum levels of LH only (unpublished results of NMB).

The quantitative analysis of the seminiferous epithelium at stage VII of the cycle reveals that GnRH antagonist reduces the number of mPSc and 7Sd significantly. Theoretically the pachytene spermatocyte : spermatid ratio should be 1:4 (Clermont and Morgenstaler 1955), but the 1:3.31 in our control rats indicates 17.25% spermatid degeneration. This ratio became 1:2.46 in GnRH antagonist treated rats fed on 5% casein diet, indicating that during the process of spermatocyte to spermatid conversion 38.5% of the cells degenerated. Feeding on 20% casein diet, GnRH antagonist treated rats show similar degeneration as observed in anti-GnRH treated rats fed on 5% casein diet. Since testicular germ cell development up to the level of step 7 spermatids (round spermatids) and meiotic division of primary spermatocytes to spermatids are supported by FSH, reduction in the number of spermatogonia, spermatocytes and spermatids in anti GnRH treated rats fed 5% or 20% casein diet may be due to low levels of FSH. On the other hand, testosterone supplementation to GnRH antagonist treated rats elevated the weights of the sex organs but failed to restore spermatogenesis quantitatively.

Moreover, concomitant administration of GnRH antagonist and high doses of testosterone maintained sexual activity and also fertility in rats while lower doses of testosterone maintained sexual activity without restoring qualitative or quantitative spermatogenesis.

Thus our observations strongly suggest that increase in testosterone levels and sex organs weights in anti GnRH treated rats after feeding 20% casein diet possibly improve sexual activity while this insignificant rise of testosterone with respect to control, is unable to restore spermatogenesis back to normal.

Little is known about how casein-enriched diet prevents adrenocortical hyperactivity and testosterone suppression in GnRH antagonist treated rats. Biologically active peptides derived from casein during the intestinal digestion are considered to be potential modulator of various regulatory process in the body. Some bioactive peptides have been shown to behave like opioid receptor ligands and milk protein may elicit opioid effects. On the other hand pharmacological studies have shown that opioids inhibit ACTH secretion. Since glucocorticoids suppress the testicular LH receptors, decrease LH secretion in cultured pituitary cells and reduce serum levels of LH, the possibility remains that bioactive peptides of milk protein may prevent the inhibition of LH secretion by suppressing adrenocortical hyperactivity in anti GnRH treated rats and/or modulate LH action on the Leydig cells which in turn stimulates sexual activity and also fertility in rats while lower doses of testosterone maintained sexual activity .

<table>
<thead>
<tr>
<th>Table 1—Effect of casein diet and GnRH antagonist on the weights of adrenal, testis, accessory sex organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>GnRH antagonist + 5% casein</td>
</tr>
<tr>
<td>GnRH antagonist + 20% casein</td>
</tr>
</tbody>
</table>

* P<0.05 compared with control.

<table>
<thead>
<tr>
<th>Table 2—Effect of casein diet and GnRH antagonist on the relative number of germ cells per tubular cross-section at stage VII of the seminiferous epithelium cycle [values are mean ± SE of 6 animals in each group]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>GnRH antagonist + 5% casein</td>
</tr>
<tr>
<td>GnRH antagonist + 20% casein</td>
</tr>
</tbody>
</table>

* P<0.05 compared with control rats.

ASg, type-A spermatogonia; PLSc, preleptotene spermatocytes; mPSc, mid-pachytene spermatocyte; 7Sd, step-7 spermatid.
testosterone secretion while spermatogenesis remains arrested due to reduced FSH secretion.

Acknowledgement

The authors thank Dr. Sekhar Bhattacharyya, Department of Chemical Engineering, Calcutta University for his sincere Co-operation in this work. Thanks are given to Professor Arun K. Roy, Bose Institute Calcutta for helping us in Radioimmunoassay. This work was supported in part by grants from ICMR, New Delhi.

References

12. Glick D, Redlich D V & Lavine S, Fluorometric determination of corticosterone and cortisol in 0.02-0.05 milliliters of submilligram samples of adrenal tissue, Endocrinology, 74 (1964) 653.