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Effects of Chronic Luteinizing Hormone-Releasing Hormone Administration on Gonadotropin Dynamics of Adult Male Rats (40966)[1]

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Abstract. The studies reported herein were conducted to determine the sequential effects of chronic luteinizing hormone-releasing hormone (LHRH) or D-Trp^6–Pro^9-NEt-LHRH (LHRH$_a$) administration on LH, FSH, and T dynamics. Adult male rats (n = 6/group) were injected subcutaneously with 1 μg of LHRH or LHRH$_a$ once daily at 8:00 AM for 1 through 7 days. The rats were decapitated at various times postinjection and their blood, pituitaries, and testes collected. When measured 24 hr after each injection, significant ($P \leq 0.01$) decreases were observed in concentrations of serum T and pituitary LH and FSH as well as testicular volume; whereas basal serum concentrations of LH and FSH were significantly ($P \leq 0.01$) elevated from Days 3 through 7 in both LHRH and LHRH$_a$-treated rats. LH was secreted in a large single peak 1 hr after each LHRH injection, increasing 54-fold above preinjection concentrations on Day 1 and lessening to a 34-fold increase on Days 3 through 7. The magnitude of the FSH response was not altered with chronic LHRH treatment. The peak responses of both LH and FSH were blunted with LHRH$_a$ treatment. When rats were castrated after 5 days of LHRH treatment, the castration-induced rise in serum FSH concentration was normal, whereas the LH rise was delayed and blunted. These data indicate that: (1) Chronic treatment with LHRH or LHRH$_a$ results in elevated basal serum gonadotropin concentrations and reduced basal serum testosterone concentrations and pituitary LH and FSH concentrations. (2) Chronic treatment with LHRH reduces the serum LH response (but not the FSH response) to subsequent LHRH injections. Furthermore, chronic treatment with essentially an equimolar dose of a potent LHRH agonist (D-Trp^6–Pro^9-NEt-LHRH) blunts both the LH and the FSH response to subsequent LHRH agonist injections. (3) FSH responses to castration are not altered by chronic LHRH treatment, whereas LH responses to castration are decreased. Treatment with a potent LHRH agonist prevents the normal rise in serum LH and FSH concentrations following castration.

The hypothalamic peptide, luteinizing hormone-releasing hormone (LHRH), is secreted into the hypothalamic–hypophyseal–portal circulation and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. Chronic administration of pharmacologic doses of LHRH or LHRH agonists to male rats results in decreases in testicular volume (1–6), *in vitro* binding of LH/hCG to gonadal tissue preparations (3, 7–10), testosterone production (1, 3, 4, 6, 11) and spermatogenesis (2, 4, 12), and elevations in basal serum LH and FSH concentrations (5, 11). The resultant inhibition of reproductive function by these peptides raises the possibility of their use as contraceptive agents. The studies reported herein are designed to more fully characterize the effects of chronic LHRH administration on the male reproductive system with particular focus on gonadotropin dynamics of intact and castrated adult male rats. The objectives of these studies were to: (1) determine the time course of changes in serum and pituitary LH and FSH concentrations and serum testosterone concentrations upon daily administration of single doses of LHRH; (2) compare the effects of LHRH treatment with those of a more potent LHRH agonist (D-Trp^6–Pro^9-NEt-LHRH); and (3) determine if chronic LHRH treatment affects the castration-

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induced rise in serum LH and FSH concentrations.

Materials and methods. Luteinizing hormone-releasing hormone (LHRH) was a gift of Ayerst Laboratories (New York) and the D-Trp^6-Pro^9-NEt-LHRH (LHRH$_a$) was kindly provided by Drs. Jean Rivier and Wylie Vale (The Salk Institute for Biological Studies, La Jolla, Calif.). The anti-LH was provided by Dr. Gordon Niswender (Colorado State University, Fort Collins, Colo.) and the LER-1056-LH for iodination was provided by Dr. Leo Reichert (Emory University, Atlanta, Ga.). The materials for the FSH radioimmunoassay and the rat LH-RP-1 were provided by the NIAMDD Rat Pituitary Distribution Program. Unless otherwise specified, all other materials used in these studies were purchased from Sigma Chemical Co., St. Louis, Missouri; Gibco, Inc., Grand Island, New York; or Fischer Scientific, Fair Haven, New Jersey.

In the first series of experiments, adult male Sprague–Dawley rats (200–225 g) were individually housed with a 12-hr-light, 12-hr-dark cycle and fed *ad libitum*. Each rat was injected once daily with a sc dose of LHRH or LHRH$_a$ (2 μg), or the vehicle for 1–7 days. The LHRH or LHRH$_a$ was dissolved in 0.1% gelatin–normal saline and the bioactivity of these peptides was confirmed by determining (i) the rise of rat serum LH resulting from a sc injection of 200 ng LHRH or LHRH$_a$, and (ii) LH release by rat pituitaries incubated with 10^{-8} *M* LHRH or LHRH$_a$. Groups of rats ($n = 6$) were decapitated at 0.5, 1, 2, or 24 hr following each LHRH injection and at 4, 8, 12, or 24 hr following each LHRH$_a$ injection and the blood and pituitaries were collected. The blood was allowed to clot on ice for 2 hr and centrifuged at 1000*g* to obtain serum which was then stored at −20°C. The pituitaries were frozen on dry ice, stored at −20°C, and later homogenized in 2 ml of 0.1 *M* phosphate-buffered saline (PBS).

In a second series of experiments the animals were treated with LHRH or LHRH$_a$ as described above for 5 days and then castrated and treated for an additional 1 day (experiment 1) or 2 days (experiment 2). The purpose of these experiments was to determine if chronic treatment with these peptides would alter the castration-induced rise in gonadotropins. The animals were decapitated 24 hr following the last injection and the blood and pituitaries were collected. In experiment 2, 24-hr urine collections were obtained.

The serum LH and FSH concentrations were determined by a micromodification (1) of the radioimmunoassays originally described by Niswender *et al.* (13) and Midgley (14), respectively. The sensitivity of the LH assay was 1.57 ng expressed as NIAMDD-Rat LH-RP-1 and the sensitivity of the FSH radioimmunoassay was 15 ng expressed as NIAMDD-Rat FSH-RP-1. The inter- and intraassay variability was less than 10% for both LH and FSH radioimmunoassays. LH and FSH were assayed in sera, PBS homogenates of pituitaries, and PBS-soluble fractions of acetone precipitates of urines.

Serum testosterone was determined by radioimmunoassay of benzene–petroleum ether extracts (recoveries were 80–95%) using a previously characterized antisera kindly provided by Dr. L. D. Loriaux of NIH, Bethesda, Maryland. The sensitivity of the assay was 20 pg and the inter- and intraassay variability was less than 8%.

The total soluble protein was measured by the method of Lowry *et al.* Testicular volume was determined by measuring the volume of water displacement. RIA data were analyzed using a program based on that of Rodbard and Lewald (15). Statistical analysis were performed using Student's *t* test.

Results. There were no statistically significant body weight or pituitary weight changes as a result of LHRH or LHRH$_a$ treatment (data not shown). Testicular volumes of rats treated with LHRH decreased only slightly from 2.8 ± 0.06 to 2.6 ± 0.06 ml after 7 days. However, rats treated with LHRH$_a$ had significantly ($P \leq .01$) reduced testicular volumes from 2.8 ± 0.01 to 1.8 ± 0.08 ml. Determination of urinary LH content showed that rats receiving LHRH excreted an average of threefold more LH per 24 hr than did saline controls (6.57 ± 1.1 vs 2.2 ± 0.3 μg/24 hr). Furthermore, when LHRH-treated rats were castrated, the

24-hr LH urinary excretion approximately doubled; whereas saline-treated rats excreted approximately fourfold more LH (data not shown).

Time response to LHRH. The time course for LHRH-treated animals was chosen based on preliminary experiments showing that following a single dose of 2 μg LHRH, serum LH and FSH concentrations were maximal at 60 and 120 min, respectively, and returned to baseline by 12 hr. The LH time responses for chronic LHRH injections are presented in Fig. 1. Serum LH concentrations were maximal at 60 min postinjection and were greatest (a 54-fold increase) after the first injection. The mean maximal increase in serum LH secretion occurring between Days 2 and 7 was 734 ± 15 mg/ml which represents a 34-fold elevation over basal levels. Therefore, the peak LH secretion was blunted with daily LHRH administration. Conversely, the basal LH concentrations, those measured 24 hr following the last LHRH injection, were significantly ($P \leq 0.01$) elevated. The basal serum testosterone concentrations, also shown in Fig. 1, were significantly ($P \leq 0.01$) decreased as a function of LHRH treatment, whereas stimulated levels of serum testosterone were essentially unaffected by treatment.

Initially (Days 1, 2, and 3), the peak FSH response (as seen in Fig. 2) occurred at 120 min postinjection. Between Days 3 and 4, the peak response shifted from 120 to 60 min and remained there through Day 7. Unlike LH, the FSH peak response to LHRH did not diminish with increased LHRH treatment, but remained constant throughout the study. Similar to LH, however, the basal FSH concentrations were significantly ($P \leq 0.01$) elevated as a function of LHRH treatment.

The pituitary concentrations of LH and

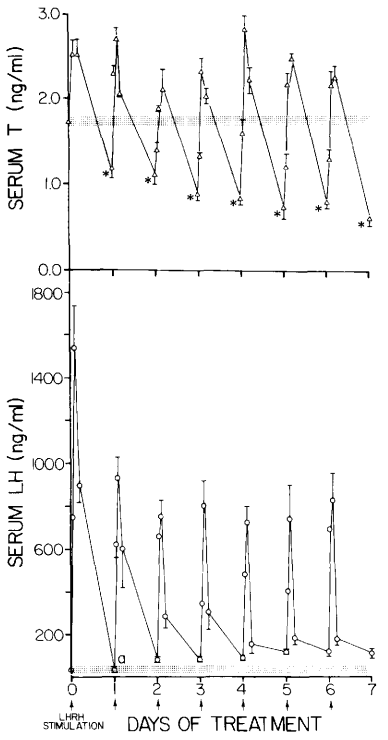

FIG. 1. LHRH (2 μg) dissolved in 0.1% gelatin–normal saline was injected sc into adult male rats once daily at 8:00 AM. The mean (±SEM) serum LH and testosterone (T) concentrations are shown for 0.5, 1, 2, and 24 hr following each daily injection. Each point represents the mean of six rats. The shaded areas represent the mean (±SEM) LH and T concentrations for saline-injected animals. Since no statistically significant differences were found between the saline group at 0.5, 1, 2, and 24 hr, the data were pooled and presented as a single mean concentration. All the mean LH concentrations (except a) are statistically greater ($P \leq 0.05$) than the saline controls. *$P \leq 0.05$ when compared to saline-treated rats.

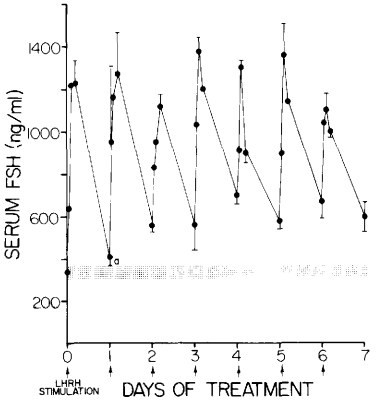

FIG. 2. The mean (±SEM) serum FSH concentrations are shown for 0.5, 1, 2, and 24 hr following each daily injection. Each point represents the mean of six rats. The shaded area represents the pooled mean FSH concentrations of saline controls. All mean FSH concentrations (except a) are statistically greater ($P \leq 0.05$) than saline controls.

FSH are presented in Fig. 3. As can be seen, concentrations of both gonadotropins were not static, but varied with each injection of LHRH. On Days 1, 2 and 3, both LH and FSH appeared to decrease by 30 min, recover by 60 min, and became maximally depressed at 120 min. With continued LHRH administration, pituitary LH concentrations were increased at 30 min and returned to postinjection concentrations thereafter, whereas FSH concentrations were less affected.

Time responses to LHRH$_a$. The time course for LHRH$_a$-treated animals was chosen because preliminary experiments showed that in response to a single 2-μg injection of LHRH$_a$, LH and FSH were each secreted in a single large peak which was maximal between 2 and 4 hr postinjection and which did not return to baseline until beyond 24 hr. Furthermore, we were concerned that the elevated basal serum concentrations of LH and FSH following chronic LHRH$_a$ administration which were previously reported by our laboratory (5) may have been due to a failure of LH and FSH return to basal levels following the last LHRH$_a$ injection. Thus, a longer time course (4, 8, 12, and 24 hr) was employed for LHRH$_a$. Serum and pituitary gonadotropin concentrations of rats injected with a potent LHRH agonist (D-Trp^6-Pro^9-NEt-LHRH) are presented in Fig. 4. The responses measured at 4 hr following the second and third injections of LHRH$_a$ were significantly ($P \leq 0.01$) less than those measured 4 hr after the first LHRH$_a$ injection. Although serum FSH concentrations had not returned to baseline levels by 24 hr after the first injection, serum concentrations for both LH and FSH returned to baseline by 12 hr postinjection thereafter. The basal serum LH and FSH concentrations (24 hr postinjection) were significantly ($P \leq 0.01$) elevated beyond 3 days and were probably not due to a residual effect of the previous LHRH$_a$ injection. The pituitary LH and FSH concentrations, as shown in the inset of Fig. 4, decreased nearly 50% by 4 hr and continued to decrease rather steadily thereafter.

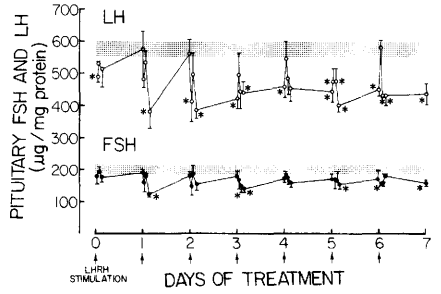

FIG. 3. Adult male rats were injected sc with LHRH (2 μg) dissolved in 0.1% gelatin–normal saline once daily at 8:00 AM. The pituitary LH (○) and FSH (●) concentrations (mean ± SEM) are shown for 0.5, 1, 2, and 24 hr following each injection. Each point represents the mean of six rats. Shaded areas represent the mean (±SEM) LH and FSH concentrations for saline-injected animals. *$P \leq 0.05$ when compared to saline-treated rats.

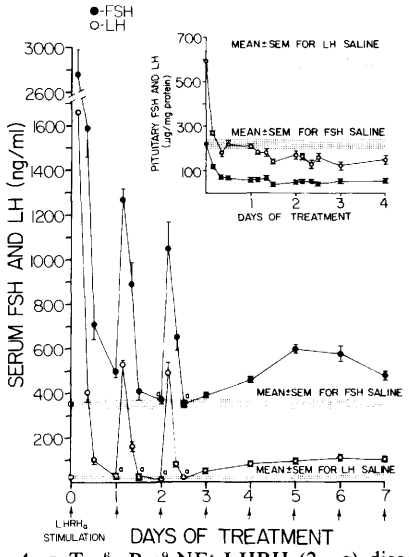

FIG. 4. D-Trp^6–Pro^9-NEt-LHRH (2 μg) dissolved in 0.1% gelatin–normal saline was injected sc into adult male rats once daily at 8:00 AM. The mean (±SEM) serum LH and FSH concentrations are presented for 4, 8, 12, and 24 hr following each daily injection given on 0, 1, and 2 days. Thereafter, samples were obtained only at 24 hr postinjection. Each point represents the mean of six rats. The insert, upper right, shows the mean (±SEM) pituitary LH and FSH concentrations. The shaded areas represent the mean (±SEM) LH and FSH concentrations for saline-treated controls. All means (except a) are statistically greater ($P \leq 0.05$) than the saline controls.

Castration studies. The results of castration experiment 1 are shown in Fig. 5. Twenty-four hours following castration of saline- or LHRH-treated rats, serum FSH

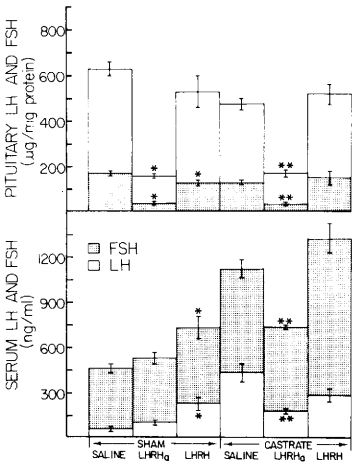

FIG. 5. LHRH or D-Trp^6-Pro^9-NEt-LHRH ($LHRH_a$) were dissolved in 0.1% gelatin–normal saline and injected sc into adult male rats once daily at 8:00 AM for 6 days. Between 8 and 10 AM on day 5, each rat was either castrated or sham operated under ether anesthesia. 24 hr following castration, rats were decapitated. The mean (±SEM) LH and FSH concentrations for serum (lower panel) and pituitaries (upper panel) are shown. Each point represents the mean of six rats. $*P \leq 0.01$ when compared to sham saline. $**P \leq 0.01$ when compared to castrate saline.

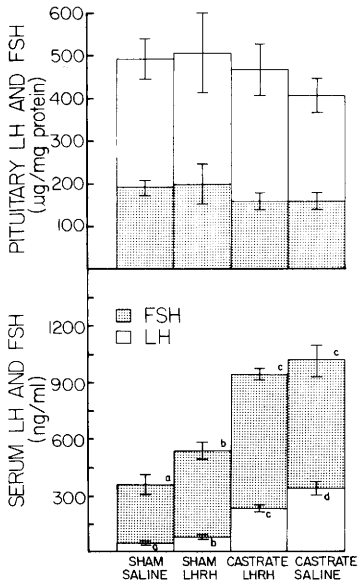

FIG. 6. Adult male rats were injected sc with 2 μg LHRH or 0.1% gelatin–normal saline for 7 days at 8:00 AM. Each rat was either castrated or sham operated under ether anesthesia the morning of Day 5. LH and FSH concentrations are shown for serum (lower panel) and pituitaries (upper panel) 48 hr following the last injection. Each mean represents the mean of six rats. Means bearing different superscript letters are significantly different ($P \leq 0.05$).

concentrations were significantly ($P \leq 0.01$) increased as compared to their respective sham controls. Serum LH concentrations of rats treated with LHRH did not significantly increase by 24 hr postcastration.

Castration of $LHRH_a$-treated rats also produced significant ($P \leq 0.05$) elevations in serum FSH concentrations as compared with sham controls, but this effect was significantly ($P \leq 0.05$) lower than the elevations observed for the saline castrates or LHRH castrates. Although $LHRH_a$-treated rats had significantly elevated serum LH concentrations at 24 hr postcastration, this response was less than half that of the castrated saline controls. The pituitary concentrations of LH and FSH were greatly decreased ($P \leq 0.001$) in $LHRH_a$-treated rats and only LH was slightly, but significantly ($P \leq 0.05$), reduced in LHRH-treated rats.

The serum and pituitary LH and FSH concentrations of castration experiment 2 are shown in Fig. 6. Serum LH and FSH concentrations in both saline- and LHRH-treated rats were elevated 48 hr following castration. However, serum LH concentrations of LHRH-treated rats were significantly ($P \leq 0.01$) lower than saline-treated castrates. No statistical differences were observed in pituitary LH and FSH concentrations between LHRH and saline-treated animals.

Discussion. Single injections of LHRH or $LHRH_a$ for 2 days were previously reported to decrease basal serum testosterone concentrations and *in vitro* testosterone production by Leydig cells (5, 6). Therefore, the reduced basal serum testosterone concentrations presented in the present study were expected, and confirm the initial work of Auclair *et al.* (1) as well as work from other laboratories including our own. It was surprising, however, to find essentially normal elevations in serum testosterone concentrations 1–2 hr following each LHRH injection in rats chronically treated with LHRH. Since it has been shown that the hCG/LH receptor levels and

serum testosterone concentrations decreased following a single injection of 1 μg LHRH (9), "receptor down regulation" may be an explanation for the 60% reduction in basal serum testosterone concentrations. For example, it has been shown that LHRH (16) or the higher doses of hCG which mimic the LH concentrations observed following LHRH treatment (16, 17) induced a stereogenic block in Leydig cells located at the site of conversion of 17-hydroxylated steroids to androgens. Furthermore, this reduction of androgen production is correlated with the degree of receptor loss. If the loss of receptors were responsible for the decreased basal concentrations of testosterone, however, elevations in serum testosterone immediately following LHRH stimulation should likewise be reduced or blunted. This was clearly not the case and further study is necessary to resolve this problem.

The peak serum LH response to repeated high doses of LHRH and the serum LH and FSH response to chronic $LHRH_a$ treatment is blunted; an effect which previously has been reported by others for LHRH agonists (11). The peak serum FSH response to repeated doses of LHRH, however, was not found to be blunted but rather to shift the maximal response from 120 to 60 min. The LH/FSH ratio was calculated using the maximal concentrations of LH and FSH following an LH injection in animals treated at least 3 days with LHRH. These ratios increased as compared to saline-treated rats and were similar to those reported by Rivier and Vale in rats chronically treated with $LHRH_a$.

The basal serum LH and FSH concentrations measured 24 hr following each injection were elevated in both LHRH- and $LHRH_a$-treated rats. The 24-hr period between injections was more than adequate for clearance of the exogenous circulating LHRH and the return of serum LH and FSH concentrations to basal levels. Since these elevations were inversely related to the declining serum testosterone concentration, it appears that this effect resulted from the removal of negative feedback of testosterone. It is possible, however, that this may reflect the increased numbers of LHRH receptors reported to occur following LHRH treatment (18).

The pituitary LH and FSH concentrations of rats treated chronically with LHRH differed greatly from those treated with $LHRH_a$. Both LH and FSH concentrations decreased dramatically within hours of the first $LHRH_a$ injection and remained depressed thereafter, never returning to preinjection levels. The LHRH-treated rats had definite suppressions and rebounds in gonadotropin concentrations following each LHRH injection. The lowest concentrations of the pituitary LH and FSH were measured 2 hr after each LHRH injection. Following the first three LHRH injections, LH and FSH concentrations returned to saline control levels by 24 hr. During Days 4 through 7, however, the 24-hr LH concentration remained depressed. It is interesting to note that during Days 1, 2 and 3 there was an apparent increase in pituitary LH and FSH concentrations at 60 min postinjection. After 4 days of LHRH treatment, this accumulation at 60 min was not seen for FSH, but continued to occur for LH with a slightly earlier time course. It is possible that this small but consistent peak of LH and FSH observed to occur at 60 min postinjection of LHRH is a "second pool" and represents newly synthesized gonadotropins. Prolonged treatment with LHRH reduces or abolishes this peak for FSH, whereas the LH response remains relatively intact.

It appears that LHRH-treated rats respond to castration by increasing gonadotropin secretion. Although the percentage increase in serum FSH concentrations following castration was less for LHRH than for saline-treated rats, the absolute concentrations for serum FSH were the same in both LHRH- and saline-treated castrates, indicating that the FSH response to castration was essentially normal. On the other hand, castration-induced rises in serum LH concentrations in LHRH-treated animals were significantly less than saline-treated rats. Treatment with the more potent $LHRH_a$ resulted in blunted castration-induced rises in both serum LH and FSH concentrations. The major differences between LHRH- and $LHRH_a$-treated

groups were the lower concentrations of pituitary LH and FSH and the blunted FSH response in the $LHRH_a$ group. It is possible that the blunted responses to castration and subsequent $LHRH_a$ stimulation of the $LHRH_a$-treated rats was simply a matter of substantial reductions in concentrations of releasable LH and FSH in the pituitary. However, a similar blunting of the LH response to castration was observed with the LHRH-treated rats who had pituitary LH concentrations similar to those of saline-treated animals, indicating that substantial pituitary LH reductions are not a prerequisite for blunting of the castration response.

These studies indicate that treatment of male rats with gonadotropin-releasing peptides of different potency and duration of action can produce different, and perhaps unique, responses of hypothalamic–pituitary–gonadal axis. For example, the $LHRH_a$-treated rats had suppressed castration-induced rises in serum LH and FSH. In contrast, the LHRH-treated rats responded to castration with a normal FSH rise and a blunted LH rise. Furthermore, the rats chronically treated with LHRH appeared to respond to daily LHRH injections with normal serum FSH elevations; whereas the rats treated similarly with $LHRH_a$ had blunted serum FSH elevations. It is not clear as to whether the differences observed between LHRH and $LHRH_a$ are the results of potency–duration differences or to activities unique in their molecular structure. If the potency–duration properties are responsible, it may be possible to select a treatment regime for either peptide which results in a tailored LH/FSH response.

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