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Effect of chronic thyroid hormone treatment on cycling, ovulation, serum reproductive hormones and ovarian LH and prolactin receptors in rats

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We studied the effect on cycling, ovulation and hormone secretion of a chronic thyroxine treatment (HT, 1 mg/kg, *s.c.*, daily, initiated at oestrus) on female rats. HT rats showed normal 4-day vaginal cycles on the first three cycles after initiation of the treatment, but on the fourth cycle had a prolonged oestrus and subsequently entered in constant di-oestrus. In spite of the normal vaginal cycles only 66%, 50%, 33% and 10% of the HT rats ovulated on cycles 1 to 4 respectively. In contrast, during cycles 2 and 3, ovulating HT rats shed a significantly greater number of ova than controls. Hormones were measured at 12.00 and 18.00 h (pre-ovulatory) on pro-oestrus and at 11.00 h on oestrus. HT ovulating rats had normal LH levels on the first two cycles, but low levels on the third one, while non-ovulating HT rats had low pre-ovulatory LH levels. Serum FSH concentrations were elevated in all the HT rats on cycles 1 and 2 and on pro-oestrus morning in cycle 3 and may have been responsible for the increase in ovulation rate. On oestrus, ovulating HT rats had higher FSH values than non-ovulating ones. Serum prolactin levels were similar to controls in all the HT rats on cycle 1, but on the subsequent cycles pre-ovulatory levels were lower than controls in all the HT rats, while values were increased in the non-ovulating HT rats on the third and fourth oestrus mornings. Pro-oestrous serum oestradiol concentrations in all the HT rats were not different from controls on cycles 1 and 2 and diminished on 3 and 4. Oestrous levels were significantly lower on the cycle 1 and only on the non-ovulating HT rats on cycle 2. Serum progesterone levels had values similar to those of FSH, with increased values in the first two cycles. Serum corticosterone levels were increased in the mornings of cycles 2 and 3, but values were normal on the fourth one. Ovarian prolactin and LH receptor mRNAs, measured on HT rats on the third pro-oestrus by Northern blotting, showed significant increases in all the majoritary molecular forms (2.5 and 7 kb for LH receptor and 0.9, 2.9–3, 5 and 10 kb for the prolactin receptor) with respect to control pro-oestrous rats. These results show a progressive disruption of cycling, ovulation and hormonal secretion after the initiation of a chronic thyroid hormone treatment in rats, which eventually lead to an anovulatory state. These results may be of importance for the interpretation of the reproductive disfunctions provoked by hyperthyroidism in women.

Keywords: hyperthyroidism; thyroxine; ovulation; cycling; prolactin; LH

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Introduction

Thyroid disorders have been implicated in a variety of reproductive problems, including ovulatory failures, preterm delivery, miscarriages and cycling abnormalities (Hagino, 1971; Freeman *et al.*, 1976; Thomas & Reid, 1987; Becks & Burrow, 1991; Rosato *et al.*, 1992a).

Untreated hyperthyroidism produces menstrual irregularities in women (Thomas & Reid, 1987) and higher incidence of preterm deliveries and foetal abnormalities have been reported in hyperthyroid pregnancies (Becks & Burrow, 1991). Hyperthyroidism induced in rats by daily injections of thyroid hormone induces a decrease in the preovulatory LH surge (Freeman *et al.*, 1976). In a previous study we found out that in pregnant animals, a chronic thyroid hormone treatment (1 mg/kg daily) produced an advancement in luteolysis, with concomitantly earlier delivery and lactogenesis, as well as impairments in lactation and maternal behaviour (Rosato *et al.*, 1992a). On the other hand, the metabolic effects of the same thyroid hormone treatment were much lower in term pregnant rats when compared with the same treatment given to virgin animals (Rosato *et al.*, 1992b), indicating that gestation produces an attenuation of the effects of hyperthyroidism.

The hypothyroid state in the rat produces a diminution in the number of ova shed during the ovulatory cycle (Hagino, 1971). In immature rats, hypothyroidism induces altered sensitivity to gonadotropins and a failure in ovulation in spite of increased FSH binding, leading to polycystic ovaries (Coppmann & Adams, 1981). Conversely, thyroxine is necessary for adequate luteinization in rats (Channing *et al.*, 1976), ovulation and corpus luteum formation in women (Maruo *et al.*, 1992a) and it synergises with FSH to induce differentiation of granulosa cells (Maruo *et al.*, 1987).

We have found previously that the hyperthyroid pregnant rats carried a significantly greater number of embryos than euthyroid ones (Rosato *et al.*, 1992a) suggesting the possibility of a hyperovulation. In the present work we have studied the effect of hyperthyroidism, induced by the same treatment used previously (1 mg/kg thyroxine, daily) on the evolution of the oestrous cycles, the reproductive hormonal patterns and ovarian LH and prolactin receptors in rats, in order to determine further the effects of hyperthyroidism on reproductive cycles.

Results

Effect of chronic thyroid hormone treatment on vaginal cycles and ovulation

After the initiation of the thyroxine treatment the rats continued to cycle normally with four day cycles, according to the vaginal smears, up to the fourth cycle post-treatment. On the fourth cycle, all the rats had one or two additional oestrus days and thereafter entered into constant di-oestrus.

Groups of rats that had been bled on the previous pro-oestrus afternoon were sacrificed on the morning of oestrus to determine the ovulation rate and correlate it with the pre-ovulatory hormone secretion. Cardiac puncture did not affect ovulation rate in the control groups, since all the rats ovulated. Table 1 shows that there was a progressive diminution of the number of rats ovulating as the number of cycles after the beginning of thyroxine treatment increased, with only one rat out of 10 ovulating on the fourth cycle. Statistical analysis of these data using the generalized linear model of the S plus program (McCullagh & Nelder, 1991; Hartie & Pregibon, 1992) gave a significant ($P = 0.01$) decrease in ovulation rate with time after the beginning of the treatment that fitted to a linear curve. There was no modification in the average number of ova shed per ovulating rat in the first cycle, while in the second cycle the HT rats could be divided into two groups, 10 rats with significantly increased ovulation rate (more than 10 ova per rat), and four rats that ovulated less than four ova. During the third cycle, the thyroxine-treated (HT) rats that ovulated also had a significantly greater number of ova than the controls. In all the groups the ovaries of the non-ovulating HT rats possessed larger follicles than the ovulating ones.

Effect of chronic thyroid hormone treatment on preovulatory hormone secretion

We measured the serum concentrations of LH, FSH, prolactin, progesterone, oestradiol and corticosterone at different times on pro-oestrus and oestrus in control rats and during cycles 1–4 in the HT rats. The 18.00 h pro-oestrus and 11.00 h oestrus samples of the HT rats were divided in ovulating and non-ovulating groups. In order to have sufficient amounts of serum to allow for the oestradiol determination, the 12.00 h pro-oestrus samples were obtained by decapitation, preventing us from differentiating these groups into ovulating and non-ovulating rats.

Table 1 Effect of chronic thyroid hormone treatment (HT, 1 mg/kg, s.c., daily) on ovulation in the subsequent cycles in virgin rats

	No of ovulating rats[a]	*No of ova per rat*[b]
Controls	9/9	8.7±0.9 (9)
HT 1st Pro-oestrus	9/13	9.9±1.6 (9)
HT 2nd Pro-oestrus[c]	10/28	14.6±0.8 (10)*
	4/28	3.0±0.6 (4)*
HT 3rd Pro-oestrus	7/21	12.1±0.5 (7)*
HT 4th Pro-oestrus	1/10	10

Control rats were treated with saline. [a]Number of ovulating rats/number of rats. [b]Results are expressed as the mean ± SEM of the number of rats indicated within parenthesis. [c]During the second proestrus 10 rats ovulated more than 10 ova and four less than four ova. *$P<0.05$ compared with controls (ANOVA and least significant difference between means test (Snedecor & Cochran, 1967)

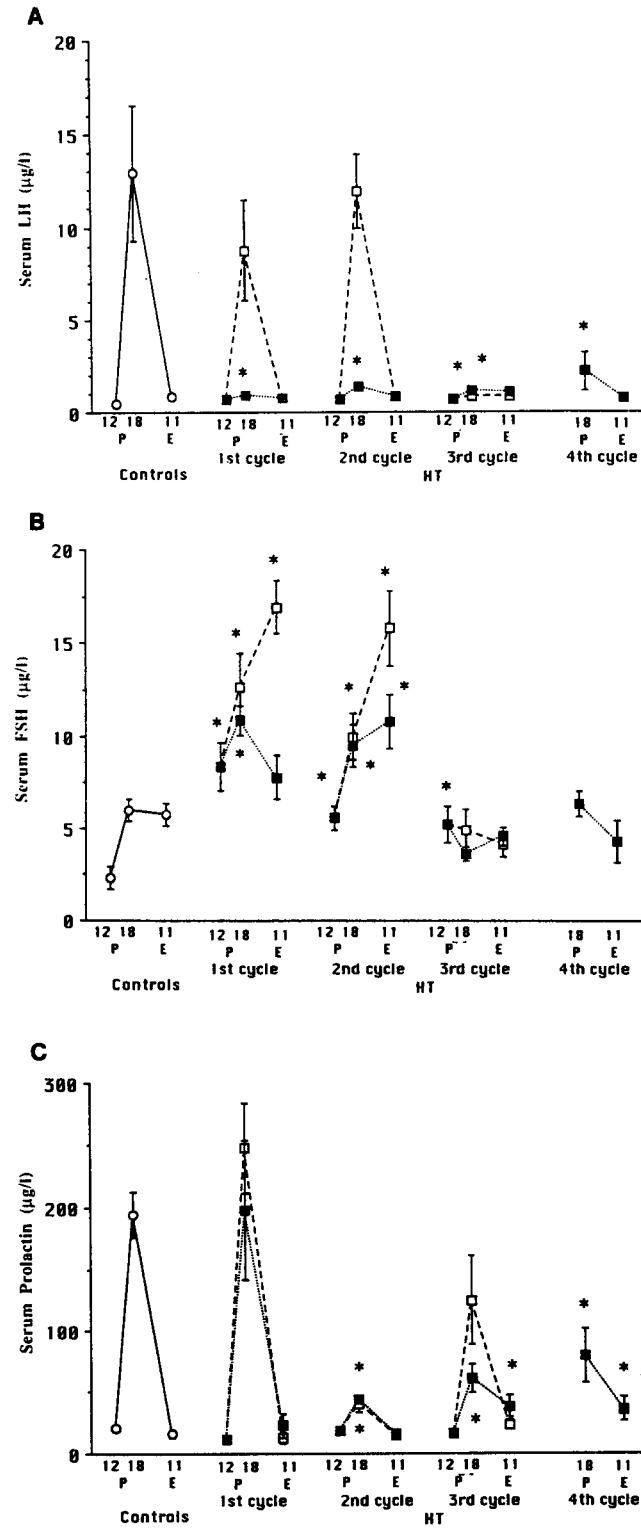

Figure 1 Serum LH (A), FSH (B) and prolactin (C) concentrations in control (○) and thyroxine-treated (□,■) rats at 12.00 h and 18.00 h on pro-oestrus (P) and 11.00 h oestrus (E) in the first four cycles after initiation of thyroxine-treatment (1 mg/kg daily). At 18.00 h pro-oestrus and 11.00 h oestrus the thyroxine-treated rats could be divided into ovulating (□) and non-ovulating (■) groups. Results are expressed as the means ± SEM (the error bar is not shown in those cases where it is smaller than the size of the corresponding symbol); the number of rats for the different pro-oestrus afternoon and oestrus groups are the same as given in Table 1; on the pro-oestrus morning groups there were 8 rats per group. *$P<0.05$ compared with respective control group (ANOVA and least significant difference between means test (Snedecor & Cochran, 1967)

During the first and second cycles, preovulatory serum LH levels were not different from controls in the ovulating HT rats, while in the non-ovulating ones serum LH levels were basal (Figure 1A). In the third and fourth cycles, ovulating and non-ovulating HT rats had low preovulatory LH levels (Figure 1A). Serum FSH levels, in contrast, were elevated during the first and second cycles and in the morning of pro-oestrus in the third cycle in all the HT rats (Figure 1B). Furthermore, in the morning of the first and second oestrus, the serum FSH values in the ovulating HT rats were significantly higher than in the non-ovulating ones (Figure 1B). In both groups of HT rats, serum prolactin levels were similar to controls in the first cycle, were diminished on the second cycle to a similar degree, and on the third cycle ovulating rats had an almost normal preovulatory prolactin peak, while the non-ovulating ones had lower values (Figure 1C). On the morning of the third oestrus and on the fourth cycle the non ovulating rats had increased values during the mornings and decreased values on the afternoon of pro-oestrus with respect to the control group (Figure 1C).

Serum oestradiol levels could be measured only on the serum samples obtained by decapitation, at 12.00 h on pro-oestrus and at 11.00 h on oestrus. During the first two cycles the pro-oestrous levels in the HT rats were not different from controls (Figure 2A), but the oestrous levels were significantly lower in both groups of HT rats on the first cycle and only in the non-ovulating ones in the second cycle. Since the values at 12.00 h on pro-estrus were obtained by decapitation, they are composed of values of animals that would or would have not ovulated, and some differences may have been masked. During the third and fourth cycles all HT rats had significantly diminished oestradiol levels, with the exception of the non-ovulating HT rats on the third oestrus (Figure 2A).

Serum progesterone concentrations in HT rats were significantly higher than controls in the mornings of pro-oestrus and oestrus during the first two cycles, while pre-ovulatory peak levels were similar to controls in the ovulating rats and lower than controls in the non-ovulating ones (Figure 2B). In contrast, during the third cycle, values were similar or lower than controls and no pre-ovulatory peaks were observed (Figure 2B).

Serum corticosterone levels in all the groups showed a pronounced increase in pro-oestrus afternoon when compared with all the morning values, reflecting the pronounced circadian variations of this hormone (Figure 2C). The concentrations were similar to controls in both groups of HT rats until the second pro-oestrus (Figure 2C), but on the second and third oestrus and third pro-oestrus morning values were significantly increased in the HT rats with respect to controls (Figure 2C).

Effect of chronic thyroid hormone treatment on ovarian LH and prolactin receptor mRNAs

Hybridization of Northern blots from ovarian mRNAs of control and HT rats on the third pro-oestrus with the LH receptor probe showed two majoritary bands at 2.5 and 7 kb and feebler bands at 1.8 and 4.2 kb (Figure 3A). Densitometric analysis of the two

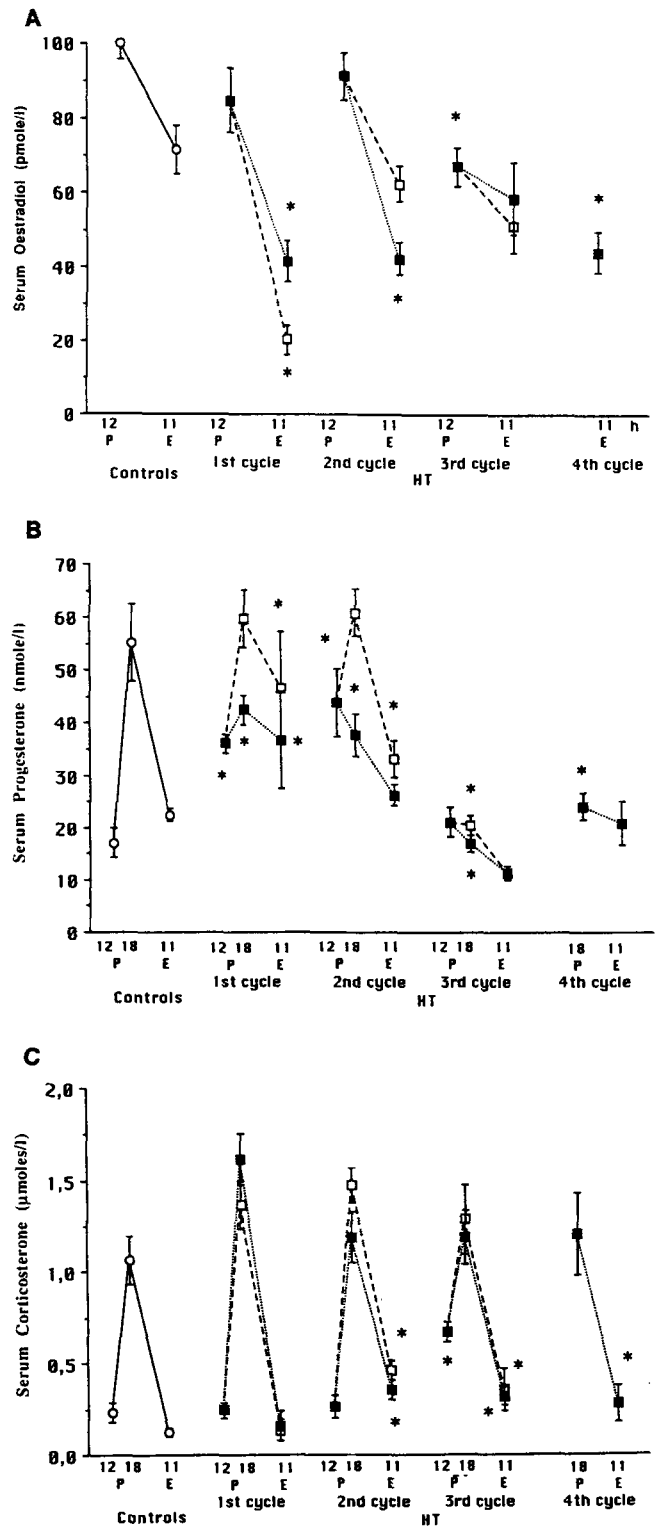

Figure 2 Serum oestradiol (A), progesterone (B) and corticosterone (C) concentrations in control (○) and thyroxine-treated (□,■) rats at 12.00 h and 18.00 h on pro-oestrus (P) and 11.00 h oestrus (E) in the first four cycles after initiation of thyroxine treatment (1 mg/kg daily). At 18.00 h pro-oestrus and 11.00 h oestrus the thyroxine-treated rats could be divided into ovulating (□) and non-ovulating (■) groups. Results are expressed as the means ± SEM (the error bar is not shown in those cases where it is smaller than the size of the corresponding symbol); the number of rats for the different pro-oestrus afternoon and oestrus groups are the same as given in Table 1; on the pro-oestrus morning groups there were eight rats per group. $P<0.05$ compared with respective control group (ANOVA and least significant difference between means test (Snedecor & Cochran, 1967)

majoritary forms showed significative increases in the HT rats (Figure 3B).

Figure 4 shows the pattern of prolactin receptor mRNAs in ovaries from control and HT rats. Hybridization of total RNA Northern blots with a probe coding for the extracellular part of the receptor gave signals at 0.9, 2.9–3, 5 and 10 kb (Figure 4A). Chronic thyroid hormone treatment induced significant ($P<0.05$) increases in all of the mRNA forms (Figure 4B).

Discussion

The present results show a progressive disruption of cycling and hormonal secretion after the initiation of a chronic thyroid hormone treatment on the day of oestrus in 4-day cycling rats. There was a progressive diminution in the proportion of rats ovulating, ending in a cessation of ovulation and cycling after the fourth cycle. Surprisingly, although a considerable number of rats did not ovulate during the first to third cycles, the rats showed apparently normal, 4 day vaginal cycles up to the fourth cycle, when they showed a prolonged oestrus followed by a constant di-oestrus and interruption of cycles. It is well known that oestrogens are responsible for vaginal cornification. The non-ovulating HT rats had apparently normal preovulatory oestradiol increases in the first two cycles but significantly lower oestradiol concentration in the morning of oestrus. The diminished levels during the third and fourth cycles were accompanied with decreased progesterone concentrations (which oppose oestrogen action at the vaginal level). Thus, the variation in oestrogen levels along with the changes in progesterone may well explain the continuation of the cycles at the vaginal level in spite of an anovulatory state. During the first cycle after initiation of the thyroxine treatment 70% of the rats ovulated, shedding a number of ova similar to the controls. On the other hand, on the second and third cycles only 50 and 33% of the rats ovulated, but those rats that ovulated had a significantly increased number of ova, with the exception of the subgroup on the second cycle that had a partial ovulation (less than four ova). These last animals may have had a different sensitivity to the effects of the HT treatment, that allowed for a partial suppression of ovulation, since there were no significant differences on the hormonal values of the preceding pro-oestrus, between them and the hyperovulating rats of the same group. We have previously shown that HT pregnant rats, mated between 10 and 15 days after the initiation of treatment, bore a greater number of foetuses than normal pregnant animals (Rosato *et al.*, 1992a). The moment of mating, thus, coincided with the period of increased ovulation, thus accounting for the increased number of embryos. The increased ovulation rate during the

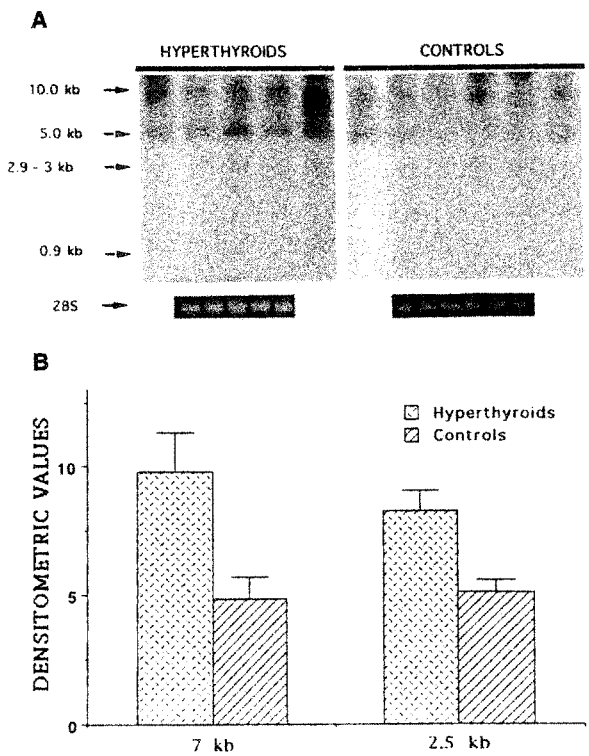

Figure 3 (A) Northern blot analysis of ovarian RNA (20 μg/lane). Samples were run on a 1.2% denaturing agarose gel, transferred to membranes and hybridized to the pLH-Receptor cDNA probe as described in Materials and methods. The lower band depicts ethidium bromide staining of the 28 S ribosomal RNA for each sample. (B) Densitometric analysis of 7 kb and 2.5 kb LH-Receptor mRNA species. The results are expressed in arbitrary units as the mean ± SEM. ($n = 5$, hyperthyroid; $n = 6$, controls). Samples from hyperthyroid rats were significantly different ($P<0.05$, Student's t test, Snedecor & Cochran, 1967) from controls

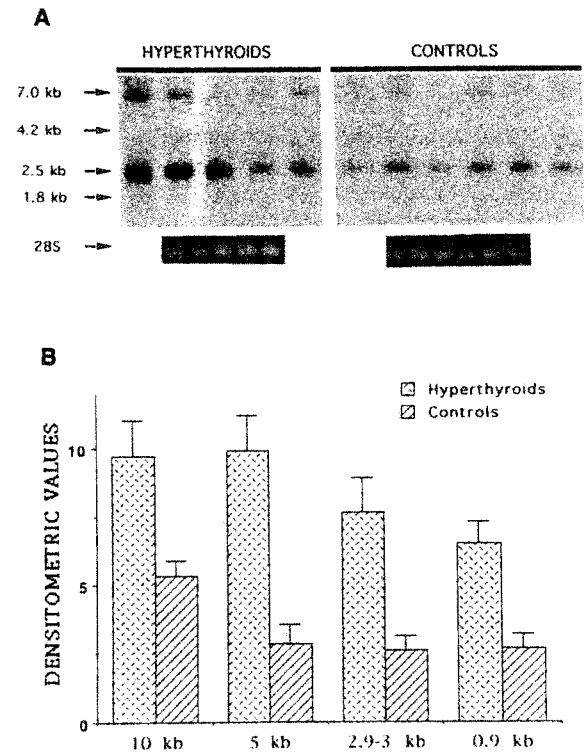

Figure 4 (A) Northern blot analysis of ovarian RNA (20 μg/lane). Samples were run on a 1.2% denaturing agarose gel, transferred to membranes and hybridized to the rat PRL-Receptor cDNA probe as described in Materials and methods. The lower band depicts ethidium bromide staining of the 28 S ribosomal RNA for each sample. (B) Densitometric analysis of major PRL-Receptor mRNA species. The results are expressed in arbitrary units as the mean ± SEM ($n = 5$, hyperthyroid; $n = 6$, controls). Samples from hyperthyroid rats were significantly different ($P<0.05$, Student's t test, Snedecor & Cochran, 1967) from controls

second and third cycles waw accompanied with markedly increased FSH serum concentrations on the previous cycles, and since FSH is mainly responsible for the recruitment of follicles, the elevated serum FSH may be responsible for the increased ovulation rate in this period. In addition, it has been shown that thyroid hormone in a physiological to moderately increased concentration synergises with FSH in inducing differentiation, gonadotropin receptor induction and steroid hormone production in granulosa cells, while very high concentrations cease to be effective (Channing *et al.*, 1976; Maruo *et al.*, 1987). Thyroid hormone receptors expression is augmented during early follicular maturation (Maruo *et al.*, 1992b). Thus, thyroid treatment at an early stage may have acted at two levels to increase ovulation rate, by elevating circulating FSH levels, and potentiating the action of this FSH at the ovarian level to induce an increase in the number of recruited follicles and also an increase in progesterone production. Thus, on the first two cycles the levels of this steroid at non-peak times (e.g. pro-oestrus and oestrus mornings) were increased in parallel with FSH, while on the afternoon of the different pro-estrus progesterone were more correlated with LH levels. Furthermore, those rats did not ovulate in the first and second cycles also failed to have increased LH levels at 18.00 h, and thus were not able to ovulate in spite of the increase in FSH. When the ovaries of these rats were examined under the dissecting microscope, they showed many mature follicles of considerable size, suggesting a partial action of gonadotropins.

The thyroid hormone treatment provoked multiple alterations in circulating hormone concentrations. The earliest changes observed were the already mentioned increases in FSH and progesterone, evident from the first cycle. Ovulating rats maintained normal preovulatory surges of progesterone, oestradiol and LH during the first two cycles and of prolactin during the first cycle only. As has been mentioned above, the increased non-peak progesterone levels in the HT rats during the first two cycles may be caused by the increased FSH concentrations, but also may be due to an interruption of luteolysis caused by the altered hormonal pattern. During the first two cycles, the preovulatory oestradiol levels were not different from the controls, but were decreased in the following oestrus. We have no explanation for this latter decrease, and of its physiological significance, if any. Our results confirm the findings of Freeman *et al.* (1976), showing a decrease in the preovulatory LH surges in rats on the second pro-oestrus after initiation of a thyroid hormone treatment, although we found that during the first two cycles the LH surges in ovulating rats were normal, and only in the third cycle both groups of HT rats had decreased LH levels. Surprisingly, one third of the rats on the third cycle ovulated in spite of low LH levels at 18.00 h . Since at this same time the ovarian LH receptors were increased, perhaps the low levels of LH were sufficient to induce ovulation in the HT animals. Nevertheless, we cannot exclude that in the ovulating HT rats an increase in LH occurred later in the evening.

Prolongation of the thyroid hormone treatment during and after the third cycle provoked further alterations in hormone secretion, abolishing the preovulatory surges of gonadotropins, prolactin and steroid hormones, while the morning serum prolactin concentrations were elevated, and finally ending in an anovulatory anoestrous state. The prolonged exposure to elevated thyroid hormone levels may have inhibited gonadotropin and altered prolactin secretion by acting at the hypothalamic level (Freeman *et al.*, 1976) and contributed to the decrease in steroid synthesis at the ovarian level by producing a desensitisation to FSH, as has been shown for very high concentrations of thyroxine in ovarian cell culture (Maruo *et al.*, 1987).

During the second and third cycle there was also an increase in the non-peak concentrations of serum corticosterone, which may have contributed further to the disruption of gonadotropin and prolactin release. It has been shown that thyroid hormone elevates circulating corticoid and hyperthyroid rats have increased basal and stress-stimulated levels of corticosterone, while hypothyroidism diminishes them (Kamilaris *et al.*, 1991).

We measured the ovarian concentrations of LH and prolactin receptor mRNAs on the third pro-oestrus afternoon as a way of appreciating the responsiveness of the ovary to the preovulatory circulating gonadotropins. In this cycle all the HT rats had abnormal ovulation, namely, hyperovulation in one third of them and none on the rest. The HT rats had increased LH and prolactin receptor gene expression. As stated above, the increased LH receptor expression may account for the ovulation in spite of decreased LH levels. The increase in LH receptor mRNAs could be indirect, produced by the combination of increased FSH levels, which regulate LH receptor expression (Segaloff & Ascoli, 1993), and potentiation of the FSH action by thyroid hormone (Maruo *et al.*, 1987). On the other hand, the increased prolactin receptor mRNA could be a direct effect of the treatment, since thyroid hormone increases prolactin receptors in the mouse mammary gland (Bhattarchaya & Vonderhaar, 1979), female rat liver (Gelato *et al.*, 1975), and male rat kidney and prostate (Marshall *et al.*, 1979; Tiong *et al.*, 1992). In contrast with our results, Tiong *et al.* (1992) reported no significant changes in prolactin receptor mRNAs in female rat tissues, among them the ovary, after a three week treatment with thyroid hormone. Most probably the lower dose used, the longer duration of the treatment, plus the fact that these authors worked with pooled tissues and did not apparently take into account the stage of the oestrous cycle when the samples were taken, may account for the difference, since ovarian prolactin receptor mRNA concentrations change significantly during the oestrous cycle (Clarke *et al.*, 1993).

In conclusion, a chronic hyperthyroid state induced by daily thyroxine injections provoked a complex and progressive alteration in the reproductive hormone secretion patterns, with precocious and transient increases in FSH, non-peak progesterone levels and increased ovarian prolactin and LH receptors. The combination of these alterations may have been the cause of the temporary increase in ovulation. Subsequently there was a progressive disappearance of the preovulatory pituitary and steroid hormone surges leading finally to an anovulatory state and the interruption of cycling which was accompanied with elevated basal corticosterone. The continuation of the

vaginal cycles in spite of a presumed anovulatory state may have been due to a maintenance of the ratio between oestradiol and progesterone during the cycle, in spite of altered concentration of these hormones. Some of the observed effects may have been a primary consequence of the elevated thyroid hormone levels, such as the extinction of the LH surges and the increases in serum FSH and in ovarian receptors, which in turn may have caused the further derangements. Since severe thyrotoxicosis in women provokes menstrual irregularities and eventual amenorrhea when untreated (Thomas & Reid, 1987), our results may be valuable in the interpretation of the effects of hyperthyroidism in human reproduction.

Materials and methods

Animals

Adult female Wistar rats, 3 to 4 months old, weighing 200–220 g originally at the onset of treatment and with 4 day cycles (checked during the preceding three cycles), were used. The rats were housed in an animal room with a 14 h light regimen (06.00–20.00 h) and controlled temperature (22–24°C); rat chow and tap water were available *ad libitum*. Hyperthyroidism (HT) was induced by daily *s.c.* injection with 1 mg/kg BW L-T_4 (generous gift from Glaxo, Buenos Aires, Argentina) dissolved in 0.9% NaCl alkalinized with NaOH to pH 9, commenced on the day of oestrus. Control rats were injected daily with saline. Vaginal smears were taken daily.

Groups of HT rats on the days of the first, second, third or fourth pro-oestrus after the commencement of treatment or control rats on the third pro-oestrus, were bled by cardiac puncture at 18.00 h performed in the conscious rat and sacrificed on the following morning (oestrus) between 10.00 and 11.00 h. The ovaries were dissected and the number of ova present on the oviducts determined under a dissecting microscope. Trunk blood was collected and serum separated and stored at −30°C for RIA of progesterone, prolactin, LH, FSH, oestradiol and corticosterone. Other groups of control or HT rats were sacrificed at 12.00 h on the day of pro-oestrus on each cycle of the treatment period, trunk blood was collected and serum separated for hormone determinations.

Other groups of HT or control rats were sacrificed at 18.00 h on the third pro-oestrus and the ovaries excised and stored immediately at −80°C for preparation and analysis of the mRNAs.

Hormone determinations

Prolactin, LH or FSH were measured by double antibody radioimmunoassay using materials generously provided by the NIADDK (Dr S Raiti, NIADDK Rat Pituitary Hormone Distribution Program). The hormones were radioiodinated using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat prolactin RP-3 or rat LH or FSH RP-2 standard preparations. Assay sensitivity was 0.5 μg/l serum and the inter- and intra-assay coefficients of variation were less than 10% for both hormones.

Serum progesterone was measured in 10 μl aliquots of unextracted sera using a radioimmunoassay developed in our laboratory (Bussmann & Deis, 1979) with an antiserum raised in rabbits against progesterone-6-CMO-bovine serum albumin conjugate (Steraloids Inc., USA). Assay sensitivity was less than 70 fmol/tube and the inter- and intra-assay coefficients of variation were less than 10%. Serum oestradiol was measured by radioimmunoassay after extraction of duplicate 0.5 ml serum aliquots (only the samples obtained by decapitation allowed enough serum for this determination) with diethyl ether and passage through an Sephadex LH 20 column as described by Butcher *et al.* (1974). Serum corticosterone was measured in the corresponding LH 20 fraction for the decapitation samples of after extraction and LH 20 separation of duplicate 50 μl serum aliquots. Appropriate dilution of the corticosterone samples was necessary. The antisera were raised in rabbits against oestradiol-6-CMO- and corticosterone-6-CMO-bovine serum albumin conjugates for oestradiol and corticosterone radioimmunoassays respectively. Assay sensitivity was lower than 12 fmole/tube and 70 fmole/tube for oestradiol and corticosterone respectively. Recuperation rates were higher than 90% and inter- and intra-assay coefficients of variation less than 10%.

RNA preparation and Northern blots

Total RNAs were prepared from whole ovaries using the guanidinium isothiocyanate/acid phenol method (Chomczynski & Sacchi, 1987) as modified by Puissant and Houdebine (1990). The RNAs (20 μg) were denatured in 50% formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1.2%(w/v) agarose gels containing 2.2 M formaldehyde and blotted on a Zeta-Probe filter (Bio-Rad). The RNAs were fixed to the membrane by U.V. irradiation. The hybridization was carried out in 500 mM phosphate buffer-7% SDS (Mahmoudi & Lin, 1989) at 65°C. The membranes were washed in 4 × SSC-0.5% SDS and exposed to Hiperfilm (Amersham) using two intensifying screens at −70°C. Densitometry analysis was performed by laser densitometry scanning (Ultroscan, Pharmacia-LKB, Piscataway, NY).

cDNA probes

The filters were hybridized with the following probes: the 1–1200 bp 5′ coding cDNA of the porcine LH receptor (Loosfelt *et al.*, 1989); and the −207/1427 bp cDNA of rat prolactin receptor (Boutin *et al.*, 1988). DNA fragments were labelled by random priming with [^{32}P]dCTP (Sambrook *et al.*, 1989).

Statistics

Statistical analysis was performed using Student's *t* test or one-way analysis of variance followed by the least significant difference between means test when more than two means were compared (Snedecor & Cochran, 1967). Differences between means were considered significant at the $P<0.05$ level. Analysis of the ovulation rate was performed using the generalized linear model with binomial error and logit link of the S plus program (McCullagh & Nelder, 1991, Hartie & Pregibon, 1992).

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