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Studies on regulation of chorionic gonadotropin secretion in primates

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Abstract. The regulation of secretion of chorionic gonadotropin in primates has been studied using both *in vivo* and *in vitro* models. *In vivo* studies using the pregnant bonnet monkey revealed that at the doses tested, the administration of progesterone or estradiol 17β in combination or alone did not result in any appreciable change in the duration or magnitude of serum chorionic gonadotropin levels. However, administration of lutropin-releasing hormone by intravenous route resulted in significant increase in chorionic gonadotropin levels within 30–60 min and the extent of stimulation seemed to depend on the state of pregnancy. For *in vitro* studies, explants or cells prepared from first trimester human placenta has been used. The functional integrity of these cells has been established by demonstrating the binding of [^{125}I]-labelled human chorionic gonadotropin antibody to the cells as well as the synthesis of [^{3}H]-labelled human chorionic gonadotropin. *In vitro* studies using the cells revealed that addition of lutropin-releasing hormone caused a significant increase in chorionic gonadotropin and estradiol 17 β secreted into the medium. Thus both *in vivo* and *in vitro* results suggest that lutropin-releasing hormone could be one of the factors involved in regulation of chorionic gonadotropin secretion in primates.

Keywords. Chorionic gonadotropin; placenta; lutropin-releasing hormone; regulation; primates.

Introduction

The placenta in addition to serving the function of transport of metabolites between the maternal and foetal system, also serves as an endocrine gland, by elaborating both protein, peptide and steroid hormones. Of the protein hormones elaborated by primate placenta, chorionic gonadotropin (CG) has been extensively studied, particularly in the case of the human. Available evidence indicates that the primary functions of CG is to extend the life span of the corpus luteum of fertile cycle and maintain its steroidogenic activity. In all the primates studied thus far, the appearance of CG is generally associated with implantation (Hendrickx and Enders, 1980). Serum concentration of CG reach maximal values during early pregnancy and it declines rapidly thereafter, either becoming undetectable as in macaques or maintained at low levels as in human females and a few other non-human primates (Tullner, 1974). Although considerable information has accrued over the years on the chemistry and physiology of CG, very

Abbreviations used: CG, Chorionic gonadotropin; hCG, human chorionic gonadotropin; LH, luteinizing hormone or lutropin; LHRH, lutropin-releasing hormone; mCG, monkey CG; NBMCS, new born male calf serum; MTP, medical termination of pregnancy; KRBG, Kreb's bicarbonate glucose.

little is known about the factors or mechanism involved in the initiation of CG production, as well as the means by which its continuous production is either shut off or substantially reduced. The present report essentially provides a brief summary of the studies initiated recently to examine the regulation of CG using both *in vivo* and *in vitro* approaches.

Materials and methods

Monkeys

For *in vivo* studies, the south Indian bonnet monkey has been used. The details of husbandry, maintenance and breeding has been described in earlier communications (Prahlada *et al.*, 1975; Rao *et al*, 1984).

Chemicals

All unlabelled steroids were obtained from Steroloids, New Hampshire, USA. Tritiated leucine (specific activity, 130–190 Ci/mmol), thymidine (specific activity, 70–90 Ci/mmol), progesterone (specific activity, 100–130 Ci/mmol), estradiol 17β (specific activity, 140–170 Ci/mmol) and [^{125}I]-were obtained from Amersham International, UK. Unlabelled thymidine, leucine and DNA were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA, collagenase from Worthington and Co., Freehold, New Jersey, USA, human chorionic gonadotropin (hCG) (CR 123) and lutropin-releasing hormone (LHRH) were gifts from NIH, Bethesda, Maryland, USA. Ovine luteinizing hormone (LH) and LHβ were kindly provided by Dr. M. R. Sairam, Clinical Research Institute of Montreal, Montreal, Canada and Dr. C. H. Li, Hormone Research Laboratory, University of California, San Francisco, USA. Tissue culture media were obtained from Himedia, Bombay and disposable culture were from Falcon Plastics, USA. New born male calf serum (NBMCS) was obtained locally and a pooled large batch was sterilised and used in all studies.

Analytical techniques

Analysis of monkey CG (mCG), progesterone, estradiol 17β, were done according to the procedures validated in the laboratory (Rao *et al*., 1984). Iodination of hCG was done according to the Chloramine T method of Greenwood *et al.* (1963). For quantitation of hCG in human placental culture samples, radioimmunoassay using rabbit anti-sera to hCG (1:40,000) and [^{125}I]-hCG was employed. The assay sensitivity was 0·l ng/ml and intra and inter-assay variations were 6·2% and 10·1% respectively.

Experimental procedures with monkeys

All injections were given at 10·00 h unless otherwise specified. Blood samples were collected from saphenous vein without using anaesthesia, serum was separated within 6 to 8h and stored at –20°C until further processing.

Isolation of cells from human placenta

First trimester human placenta (before 10 weeks of pregnancy) was collected from cases of medical termination of pregnancy (MTP) from local hospital. The tissue suspended in Kreb's Ringer bicarbonate glucose (1 mg/ml) (KRBG), pH = 7·2, was quickly transported on ice to the laboratory, washed extensively with cold KRBG to remove blood clots and villi were separated out by visual examination. The villi were minced with fine scissors and digested with collagenase (0·1 mg/ml) in KRBG for 10–15 min at 37°C with gentle shaking. After digestion, the tissue suspension was diluted with KRBG and dispersed by drawing it in and out of a sterile plastic syringe connected with a tygon tubing and allowed to settle by standing. Supernatant was carefully removed and filtered through a 100 μ nylon mesh and the process repeated several times till no tissue remained. Pooled filtrate was centrifuged at 500 *g* at room temperature, pellet was washed once with KRBG and resuspended in minimal volume of Ham F-10 medium containing antibiotics (Penicillin 100 units/ml, Streptopenicillin 100 μg/ml, Gentosporin 50 μg/ml) and 20% NBMCS. For short term *in vitro* studies, cells (1×10^6 cells/tube in 0·5 ml) were incubated with 1 μCi tritiated leucine or thymidine for 4 h at 37°C in KRBG under 95% oxygen and 5% carbon dioxide and processed for incorporation of label into protein and DNA according to procedures standardised in this laboratory (Sheela Rani and Moudgal, 1977). hCG in the incubation medium was precipitated using a highly potent antisera to hCG in goats.

Cell culture

For culture, 5×10^6 cells in 5 ml of Ham F-10 medium were plated in Falcon petri dishes and maintained under sterile conditions in Forma Water Jacketted Incubator with 5 % carbon dioxide at 37°C. Medium (containing 10 % NBMCS and antibiotics) was changed every 24 h and hCG, progesterone and estradiol 17 β in the medium were analysed by radioimmunoassay. For demonstration of [^{125}I]-labelled hCG antibody binding to cultured cells, cells were fixed with methanol, acetic acid (3:1) and incubated with [^{125}I]-labelled antibody in the presence or absence of excess unlabelled goat gamma globulin. [^{125}I]-was monitored in a PRlA's Packard Counter and β-counting was done in an LKB counter. Statistical analysis was done using Student's '*t*' test.

Results

In vivo studies in the pregnant bonnet monkey

Effect of administration of gonadal steroids on the pattern of CG secretion during early pregnancy in Macaca radiata: It is well known that pituitary gonadotropin secretion is controlled *via* feed back regulation by gonadal steroids. As the placenta produces both gonadotropins and steroid hormones (CG somatomammotropin, progesterone, estradiol 17 β and others), it was considered of interest to examine whether the administration of gonadal steroids has any effect on serum CG levels. The experimental design consisted of confirming pregnancy by monitoring CG levels on day 28 of fertile cycle, and injecting progesterone and estradiol 17 β thereafter according to protocol, described below. Our earlier studies (Rao *et al.,* 1984) have shown that CG is detectable

by about day 28 of fertile cycle, and that it rapidly reaches peak values by day 35–37 declining thereafter to undetectable levels beyond days 50–55 of fertile cycle. Since the mid-cycle estrogen and LH surges occur in these monkeys on days 8–9 and 10–11 of cycle respectively (Rama Sharma *et al.*, 1978) and the females are mated with breeder males between days 9–14 of cycle. We have considered here day 12 of fertile cycle as day 1 of pregnancy. After confirming pregnancy, the monkeys were injected at 10.00 h i.m. 1 mg of progesterone or 10 μg of estradiol 17 β or a combination of both in 0·5 ml of groundnut oil per monkey per day from day 30 to day 40 of fertile cycle. Blood samples were collected from day 30 to 60 of fertile cycle and serum CG was determined by radioimmunoassay. The results obtained in the experiment where a combination of progesterone (1 mg) and estradiol 17β (10 μg) was administered is presented in figure 1.

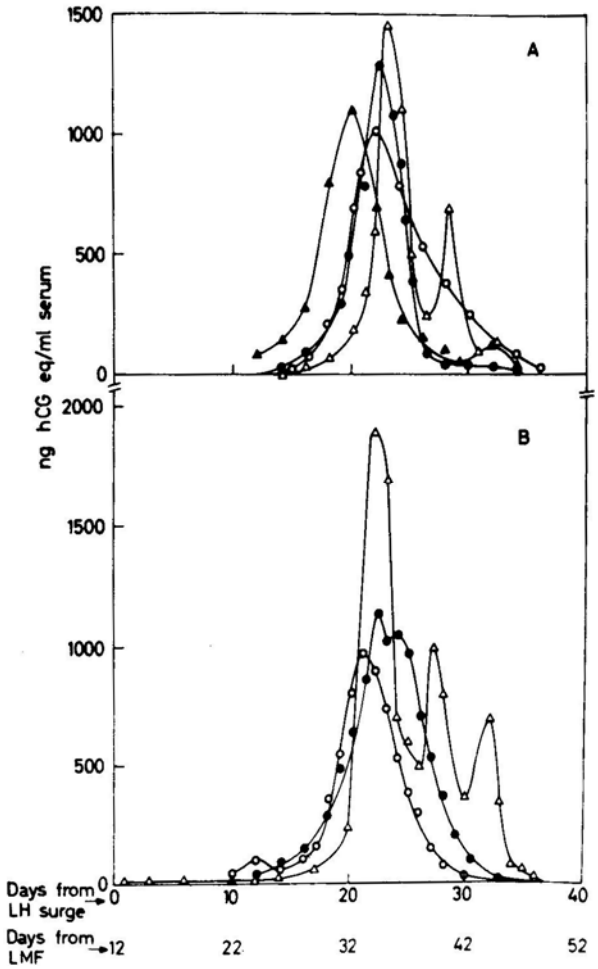

Figure 1. Effect of administration of progesterone (1 mg) and estradiol 17 β (10 μg) every day from day 30 to 40 of fertile cycle on serum CG levels in pregnant bonnet monkey. **A.** Depicts the normal profile of CG in four pregnant monkeys. **B.** Depicts the profile following administration of progesterone and estradiol 17 β in another set of three monkeys.

No significant change in the pattern or magnitude of CG levels (figure 1B) during the period under study could be seen when compared with the normal profiles (figure 1A). Essentially similar results were obtained even where each of the steroid hormones were injected separately. The present dose was selected taking into consideration the average early pregnancy in the bonnet monkey. It appears necessary that more studies have to be carried out by injecting different quantities of progesterone and estradiol 17 β before a definitive conclusion can be reached.

Effect of LHRH administration on serum levels of CG during early pregnancy: The role of LHRH in modulating pituitary gonadotropin secretion is well documented, using a variety of experimental animals. Recent studies have provided evidence that LHRH like material is present in the human placenta (Siler-Khodr and Khodr, 1978). It has also been shown that it is biosynthesised in the placenta (Khodr and Siler-Khodr, 1980) and that addition of LHRH to cultures of human placenta results in an increase in CG released into the medium (Siler-Khodr and Khodr, 1981). In view of this it was considered worthwhile examining the effects of LHRH administration in an in *vivo* model. Two different regimens of LHRH were used. The first one consisted of administering a small dose (10 μg) of LHRH by i.m. route in 0·2% gelatin every day from day 30 to day 36 of fertile cycle and analysing daily serum samples for CG. The other approach consisted of giving a bolus of 100 μg of LHRH by i.v. route on different days of fertile cycle and analysing blood samples collected at close time intervals for serum CG. The results of these studies are presented in table 1 and figure 2. It can be seen that administration of a low dose of LHRH chronically had no effect on serum CG levels (table 1) as judged by the levels on day 37 of fertile cycle (normally CG levels reach maximal values by this day). However, use of a large dose of LHRH resulted in a 2 to 3 fold increase in serum CG levels within 30–45 min (figure 2). Even here, stimulation was restricted to days 21–40 of pregnancy, no stimulation was seen when LHRH was administered during days 45–50 of fertile cycle. In order to rule out the possibility that the observed increase in CG is not due to interference in the mCG assay employed by pituitary LH released in response to LHRH, a control experiment was carried out. In this, non-pregnant cycling females were given 100 μg of LHRH on day 28 of cycle (corresponding to day 16 of pregnancy) and blood withdrawn at different times was

Table 1. Efect of administration of low doses of LHRH chronically on serum concentration of mCG in bonnet monkeys.

Group	CG concentration ng hCG eq/ml on day 37 of fertile cycle Mean ± S.E.M.
Control (4)	1242 ± 214
LHRH (3)	1325 ± 301*

l0 μg of LHRH was injected by i.m. route every day from day 30 to day 36 of fertile cycle in 0·2 % gelatin,
n of each group is indicated by number in parenthesis.
* Not significantly different from control.

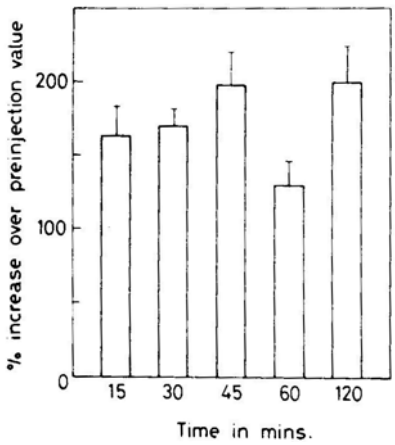

Figure 2. Efect of administration of 100 μg of LH RH by intravenous route to pregnant monkeys.

The stage of pregnancy in animals employed (n = 5) ranged from 21 to 40 days. Blood samples were collected prior to injection and at stipulated intervals following injection and serum mCG monitored by radioimmunoassay. Preinjection values were considered as 100% and any change following injection of LHRH expressed as percentage of preinjection value.

analysed for mCG. Inclusion up to 300 μl of serum from these animals did not result in any interference in the mCG radioimmunoassay indicating the specificity of the assay employed. This essentially authenticated the results obtained with LHRH in pregnant monkeys.

In vitro studies using human placental tissue

As the placental tissue collected at MTP is generally contaminated with blood clots and uterine tissue, one of the first requirements before using the tissue for experiments was to establish the identity and functionality of the cells isolated from the tissue. This was ascertained by demonstrating the presence of hCG as judged by binding of [^{125}I]-labelled antibody to intracellular hCG as well as precipitation of [^{3}H]-leucine labelled/hCG synthesised by the cells. The data provided in table 2 shows that the cultured cells bound significant amount of [^{125}I]-labelled hCG antibody over the

Table 2. Localisation of hCG in cultured cells isolated from first trimester human placenta.

Group	CPM[^{125}I]-hCG antibody bound per plate
Non-specific	155510 ± 18335
Specific	347167 ± 29066*

Each value is Mean ± S.E.M. of 4 observations.
* P <0·005.
Cells were cultured for 15 days as described in methods. Medium removed, cells fixed with methanol-acetic acid (3:1) followed by saline wash. In Group I, plates were incubated in the presence of excess of unlabelled goat gamma globulin and [^{125}I]-labelled gamma globulin prepared from serum of goats immunised with hCG; while Group II was incubated only with [^{125}I]-labelled anti hCG antibody.

controls and the data presented in table 3 shows the synthesis of [^{3}H]-leucine labeled hCG by the cells. Also the data presented in table 4 shows the viability of cells as judged by its ability to incorporate [^{3}H]-leucine into protein and [^{3}H]-thymidine into DNA.

Table 3. Demonstration of hCG synthesis by cells isolated from first trimester human placenta using short term incubation conditions.

Group	CPM[^{3}H]-leucine incorporation per mg protein
Addition of non-immunised goat serum	12026 ± 1192
Addition of hCG immunised goat serum	25317 ± 2761*

Incubation was done at 37°C under 95 % oxygen and 5 % carbon dioxide for 6h in the presence of [^{3}H]-leucine. Medium was processed for labelled hCG synthesised by addition of normal goat serum or goat antiserum to hCG.
Each value is a Mean ± S.E.M. of 3 observations.
* $P < 0{\cdot}025$.

Table 4. Experiment to demonstrate the viability of placental cells isolated from first trimester human placenta.

Group	[^{3}H]-Leucine incorporated into protein CPM/mg protein	[^{3}H]-Thymidine incorporated into DNA CPM/μg DNA
Control**	6575 ± 2403	15 ± 1·78
Experimental	34929 ± 3097*	4361 ± 503*

** Cells-incubated with excess of unlabelled leucine or thymidine.
* $P < 0{\cdot}001$ compared to corresponding control.
Cells (1×10^6) were incubated at 37°C under 95% oxygen and 5% carbon dioxide for 6 h. Medium was processed for protein and DNA.

Pattern of CG, progesterone and estradiol 17β secretion by cells in culture

The data presented in figure 3 shows the pattern of CG, P and E secretion into the medium over a period of 12 days. In agreement with other studies, Belleville *et al.* (1978), there is a rapid fall in the quantity of hCG, progesterone and estradiol 17 β secreted into the medium within first 48–96 h after which a steady state is maintained indicating that the cells are in a viable state during experimental period.

Effect of addition of LHRH on CG, P and E secretion into medium

In view of the recent demonstration that the placenta also produces LHRH like material (Siler-Khodr and Khodr, 1978), it was of interest to examine the effects of

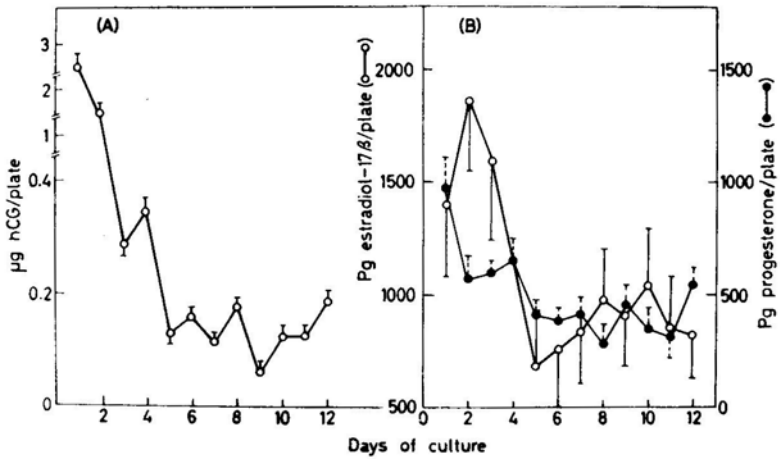

Figure 3. Pattern of secretion of CG (A), progesterone (●) and estradiol 17β (O) (B) by human placental cells in culture.

Cells were isolated and cultured as described in methods. All points are Mean ± S.E.M. of 8 observations. Values represent the quantity secreted during a 24 h period.

LHRH on CG secretion under in *vitro* conditions. Addition of LHRH (3·3 μg/ml) caused a significant increase in CG and estradiol 17β secreted into the medium (table 5). However, no effect was seen on progesterone concentration in medium. Thus the *in vitro* studies also provide additional evidence for the possible involvement of LHRH in stimulation of CG secretion.

Discussion

The regulation of pituitary gonadotropin secretion by the modulation of the gonadal steroids *via* LHRH is a well established fact. Functionally the placenta seem to exhibit characteristics of a composite hypothalamus, pituitary and gonad. In view of this it is quite justifiable to expect the placental function to be regulated by a similar mechanism.

Table 5. Effect of addition of LHRH on hCG and estradiol 17 β secretion into culture medium by human placental cells.

Group	ng hCG/plate Mean ± S.E.M.		pg estradiol 17β/plate Mean ± S.E.M.	
	Control	Experimental	Control	Experimental
Before addition of LHRH	2·13 ± 0·13	2·15 ± 0·15	375 ± 24	383 ± 16
24 h after addition of LHRH	2·20 ± 0·52	5·5 ± 0·2*	241 ± 86	1150 ± 279*

Number of plates per group = 3.

* $P < 0·05$ compared to value before addition.

LHRH (3·3 μg/ml) was added per plate and 24 h later medium analysed for hCG and estradiol 17 β, by radioimmunoassay.

This is all the more to be expected for CG as this shares considerable structural and functional homology with LH which is regulated by LHRH. However, our results show that while a negative regulation *i.e*., feed back inhibition by gonadal steroids does not seem to operate at the doses tried, LHRH seems to have a positive regulatory effect by stimulating CG production. The radioimmunoassay employed for monitoring CG values, has been validated for specificity using sera obtained from non-pregnant monkeys. The serum samples of LHRH injected non-pregnant monkeys have, as expected, been found to have increased serum LH as monitored by the mouse leydig cell assay (unpublished results). It should be pointed out that several studies have reported that CG is not detectable in pregnant rhesus monkeys beyond day 50–65 of fertile cycle (Hodgen, 1980). All the same Siler-Khodr (1979) report that administration of a relatively large dose of LHRH to pregnant rhesus monkeys on day 100 of gestation results in an increase in CG within 15 min. The present studies have been carried out using much lower dose of LHRH and monkeys at a stage of pregnancy when CG is normally secreted.

In vitro studies using placental cell cultures have also shown that LHRH stimulates CG, and estradiol 17β secretion into medium. This is in agreement with the studies carried out by Ashitaka *et al.* (1980) and Siler-Khodr and Khodr (1981). In the present studies, analysis of hCG has been carried out by using an antiserum whose specificity towards α and β subunits was not fully established. Perhaps the use of a characterized antisera to hCG would have provided more valuable information as the available studies on the cell-free synthesis of hCG indicates that the synthesis of β subunit is rate limiting (McQueen *et al.*, 1978). Although our results suggest that LHRH stimulates CG secretion in placenta, it is not clear from these studies whether it stimulates synthesis of both subunits or only one of them or just the release. However, Ashitaka *et al.* (1980) report that while dibutyryl cyclic AMP stimulates only the release, LHRH may stimulate both production and secretion of hCG and its subunits. Studies of Currie *et al.* (1981) have also shown that there are specific receptors for LHRH in human placenta. Das and Talwar (1983) have shown that administration of LHRH agonist during early pregnancy resulted in termination of pregnancy in baboons. Our preliminary studies (unpublished observation) have shown that administration of antiserum to LHRH resulted in a decrease in serum CG levels. All the above cited evidences as well as the results described here clearly suggests that LHRH may be one of the important hormones involved in regulation of CG secretion. It has been suggested that LHRH is produced by the cytotrophoblast and acts on the syncytiotrophoblast which produces both steroids and peptide hormones. Thus while the pregnant monkey model described here provides a convenient way of assessing the activity of LHRH antagonists *in vivo,* the human placental system could perhaps be used for assessing quickly the *in vitro* activity of LHRH antagonists.

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