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The Effects of Prolactin and Gonadotropin on Luteal Function and Morphology in the Cyclic Golden Hamster

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Abstract. The present study was conducted to evaluate the endocrinological effects of the pituitary on luteal maintenance and regression in the cyclic golden hamster (*Mesocricetus auratus*). After hypophysectomy (Hypox) at 0900 h on day 1 of the estrous cycle (the day of ovulation), the animals received injection of prolactin (PRL) or PRL plus equine chorionic gonadotropin (eCG). They were decapitated at 1500 h on day 3 of the cycle, and trunk blood was collected for measurement of progesterone (P4). Corpora lutea (CLs) were dissected from one ovary for DNA ladder detection by electrophoresis, determination of DNA fragmentation ratio by fluorometric measurement method and measurement of P4. The other ovary was used for histological observation. After the Hypox, the daily injection of 1 mg ovine PRL restrained the DNA fragmentation ratio and number of apoptotic cell in the CLs. The PRL treatment maintained the luteal morphology and increased the luteal P4 concentration, but not in the plasma P4 concentration. In addition to PRL, injection of 2 IU eCG after the Hypox also restrained the DNA fragmentation ratio and number of apoptotic cells in the CLs to the level of a pregnant animal. The PRL plus eCG treatment maintained the luteal morphology in the same manner as the PRL only treatment and increased not only the luteal but also the plasma P4 concentration. These results suggest that PRL restrains luteal apoptosis and maintains luteal morphology and that the combination of PRL and eCG restrains not only structural but also functional luteal regression in the cyclic hamster.

Key words: Apoptosis, Corpus luteum, Golden hamster (*Mesocricetus auratus*), Hypophysectomy, Prolactin (PRL)

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The corpus luteum (CL) is a transient endocrine organ required for normal pregnancy in mammals. The most important function of the CL is to secrete progesterone (P4). If pregnancy does not occur, the CL must regress to allow initiation of a new reproductive cycle. Luteal function and structure are regulated by luteotropic and luteolytic hormones. Luteotropic hormones maintain and activate the CL, and luteolytic hormones lead the CL to regression. Whereas the factors regulating follicle development, ovulation and luteinization are common to many mammalian species, the luteotropic and luteolytic factors are different between species [1–3].

The estrous cycle of the golden hamster lasts exactly 4 days (day 1=ovulation day), and the functional life span of the CL is limited to the first 2 days of the cycle. Serum P4 levels decrease drastically at the end of day 2 (functional luteal regression) [4]. Following functional regression, the CL shows signs of structural regression on day 3 of the cycle and vanishes by the next ovulation [5, 6]. This rapidity of structural regression is a feature of hamster luteolysis. In the rat, although the functional life span of the CL is about 2 days as in the hamster, the luteal structure persists for 3–4 cycles [1, 3]. This difference suggests that the regulatory mechanisms of structural luteal regression are different in the rat and hamster. In the cyclic rat, prolactin (PRL) is very important for structural luteal regression. In hypophysectomized rats, structural regression is

restrained, and the luteal structure remains in the ovary for several months [7]. On the other hand, in the hypophysectomized cyclic hamster, the CL regresses at the same time as in intact cyclic animals [8]. Another study showed that withdrawal of estrogen or androgen and administration of P4 do not affect the luteolytic process in the cyclic hamster [6]. Furthermore, although it is well known that prostaglandin F2 α (PGF2 α) secreted from the uterus is a strong luteolytic factor in ruminants, hysterectomy does not affect luteal regression in the cyclic rat, mouse and hamster [2]. This indicates that uterine PGF2 α is not a primary luteolytic factor in the cyclic rodent. In the pregnant hamster, hypophysectomy (Hypox) induces CL regression, and PRL plus follicle stimulating hormone (FSH) replacement restrains CL regression [9, 10]. These previous results suggest that the luteal regression of the hamster is due to the lack of luteotropic stimulation rather than to the direct effect of a luteolytic factor.

In the previous studies [8–10], the effects of hypophysectomy on luteal regression were evaluated by CL weight, diameter and histological analysis using haematoxylin and eosin staining. However, in 1990s, it was reported that apoptosis occurs in the CL during spontaneous luteal regression in many species [11–16]. In the hamster, apoptosis is also observed during luteal regression in the estrous cycle [5, 6]. Therefore, in the present study, we reevaluated the role of pituitary hormones on the CL, with a particular focus on apoptosis, in the cyclic golden hamster. Luteal apoptosis was determined by DNA fragmentation ratio and histological analysis. The effects on the luteal function were also determined by mea-

surement of the serum and luteal P4 concentrations.

Materials & Methods

Animals and treatments

Adult female golden hamsters (*Mesocricetus auratus*) were maintained under controlled temperature and lighting (lights on from 0500 to 1900 h) conditions. The 4-day estrous cycle was determined by the presence of a characteristic vaginal discharge on the morning of the day of ovulation, which was designated day 1 of the cycle. Animals with at least two consecutive 4-day estrous cycles were used in this study. The animals were divided as follows according to the treatment they received. The animals in the Hypox group (n=7) were hypophysectomized at 0900 h on day 1 of the cycle by the parapharyngeal approach under sodium pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL, USA) anesthesia. The animals in the H+PRL group (n=7) received Hypox on day 1 and then 0.5 mg ovine PRL (NIADD-o-15) was injected subcutaneously at 0900 and 1700 h on days 1 and 2 and at 0900 h on day 3; the PRL was dissolved at 500 μ g / ml in 10% (w/v) polyvinylpyrrolidone (in 0.01 M PBS, pH 8.0). The animals in the H+PRL+eCG group (n=7) received the PRL treatment plus 2 IU equine chorionic gonadotropin (eCG) dissolved in physiological saline, which was injected intraperitoneally immediately after Hypox. All animals were decapitated at 1500 h on day 3 of the cycle after Hypox. Intact animals at day 3 of the cycle (intact group; n=10) and day 3 of pregnancy (pregnant group; n=7) served as control groups (The day of sperm recovery in vaginal smears was designated as day 1 of pregnancy.). After decapitation, trunk blood was collected, and the plasma was stored at -20 C until P4 determination by RIA. The ovaries were removed and placed into cold TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0). Seven corpora lutea (CLs) were dissected using tweezers from one ovary of each hamster under a dissecting microscope. The seven CLs were weighted together, and one was stored in 1 ml 100% methanol at -20 C for measurement of luteal P4 content. The rest of the CLs were divided in half and stored at -20 C for DNA extraction. The other ovary was used for histological analysis.

Detection of DNA ladders by electrophoresis

To detect oligonucleosomal DNA ladder patterns, low molecular weight DNA fragments were extracted from CLs and electrophoresed on agarose gel. DNA fragments were isolated using the procedure described by Sellins and Cohen [17] with slight modifications. Dissected CLs were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA, pH 8.0; 0.5% TritonX-100) and centrifuged at 20,000 g for 30 min. The supernatant, containing fragmented DNA, was transferred into a new tube and digested with RNase (Qiagen, Valencia, CA, USA) and protease K (Qiagen). The DNA fragments were extracted with phenol-chloroform-isoamyl alcohol (PCI; phenol:chloroform:isoamyl alcohol=25:24:1) and concentrated by ethanol (EtOH) precipitation. At first, to confirm whether oligonucleosomal DNA fragmentation occurred in the CLs, DNA extracted from twelve CLs of the intact group were electrophoresed on 2% agarose gel at 100 V for 30 min

with DNA digest (pBR322/BamHI/MvaI). After electrophoresis, the gel was stained by ethidium bromide and viewed and photographed on a UV transilluminator. Next, to evaluate effects of pituitary hormones on luteal DNA fragmentation, DNA extracted from each group of CLs was electrophoresed on the same gel. Three CLs per an animal were used for this experiment. However, three CLs were not enough to detect ladder patterns by ethidium bromide staining, and therefore the DIG system was used to detect DNA ladder patterns in this experiment. The 3'-termini of the extracted DNA fragments were labeled with digoxigenin (DIG)-11-ddUTP (Roche Diagnostics, Rotkreuz, Switzerland) using terminal deoxynucleotidyl transferase (TdT; Roche). After labeling, PCI extraction and EtOH precipitation was performed again. The samples were electrophoresed on 2% agarose gel at 100 V for 30 min. According to the procedures for southern blotting, the electrophoresed DNA was transferred from the gel to a nylon membrane (Nylone Membranes, positively charged; Roche). Thereafter, the signals were detected using anti-DIG alkaline phosphatase-conjugated Fab antibody (Roche) and CSPD (Roche), and the membrane was then exposed to X-ray film (Fuji Film, Tokyo, Japan), which was subsequently developed.

Determination of DNA fragmentation ratio by fluorometric measurement method

To measure the quantity of DNA fragmentation, the DNA fragmentation ratio was determined by the DNA fluorometric measurement method using diaminobenzonic acid hydrochloride (DABA) [18]. For this experiment, three CLs per an animal and five animals per treatment group were used. Fragmentated DNA and intact DNA were separated using the same procedure described above and were purified by EtOH precipitation. After drying, 100 μ l of 1 N NH₄OH was added to the samples, and they were then dried completely. For DNA standards, hamster liver DNA solution was prepared by the same procedures as for luteal DNA. One ml of DABA reagent (0.3 g of 3,5-diaminobenzonic acid in 1 ml of 4 N HCl) was added to all samples, which were then incubated at 60 C for 30 min. One ml of 0.6 N HClO₄ was added to each sample, and the samples were then centrifuged at 20,000 g for 10 min. The supernatants were put on a 96-well microtiter plate. These samples were excited at 450 nm, and the fluorescence intensity was read at 530 nm using a fluorescence multi-well plate reader (CytoFluor II; PerSeptive Biosystems, Framingham, MA, USA). DNA fragmentation ratio refers to the ratio of DNA in a 20,000 g supernatant to the total DNA recovered in the 20,000 g supernatant and pellet.

Histology

One ovary from each animal used for histology was fixed in 4% paraformaldehyde (PFA) in 0.01M PBS for 24 h, embedded in paraffin, sliced serially at 4 μ m and stained with haematoxylin and eosin. To count the number of apoptotic cells and neutrophils, two fields of a CL per an animal were photographed, and two observers who were blinded to the treatments counted the number of apoptotic cells and neutrophils. For this experiment, the ovaries of four animals of each group were used. Apoptotic cells and neutrophils were recognized on the basis of their morphological features as described previously [5, 6].

Fig. 1. Mean weight of CLs on day 3 of the cycle (Intact), CLs on day 3 of pregnancy (Pregnant), CLs on day 3 of the cycle after hypophysectomy on day 1 (Hypox) and CLs treated with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) after hypophysectomy (7 CLs each). Values represent means + SEM. The values without a common letter are significantly different ($P < 0.05$).

Determination of P4 by radioimmunoassay (RIA)

Luteal P4 was extracted from one CL of each animal with 1 ml of 100% methanol at -20°C for 1 week. Plasma P4 was extracted with ether as described previously [19]. The concentrations of P4 in the extracts were measured by double-antibody RIA system using ^{125}I -labelled radioligands as described previously [19]. For this experiment, the plasma and CLs of seven animals from each group were used. Antisera against progesterone (GDN 337) were kindly provided by Dr GD Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 6.3 and 15.4%.

Statistics

All data were expressed as means + SEM. Statistical differences among the treatment groups were analyzed by one-way ANOVA followed by Duncan's multiple-range test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Effects of hypophysectomy and PRL and eCG replacement after hypophysectomy on luteal structure

The mean weights of the CLs are shown in Fig. 1. The CL weight of the Hypox group was significantly lighter than that of the other groups. Although PRL treatment (H+PRL and H+PRL+eCG) increased CL weight in a manner similar to the intact group, the CL weights of these groups were significantly lighter than that of pregnant group. There was no significant difference between the weights of the H+PRL and the H+PRL+eCG groups.

To confirm oligonucleosomal DNA fragmentation in CLs, DNA fragments extracted from twelve CLs of the intact group were electrophoresed with a DNA size marker (Fig. 2). The luteal DNA of the intact group exhibited a clear ladder pattern. The size of the smallest fragment was about 180 bp, and the sizes of other frag-

Fig. 2. Agarose gel electrophoresis of DNA extracted from 12 CLs from cyclic hamsters at 1500 h on day 3 of the cycle (right lane). The extracted DNA was electrophoresed on 2% agarose gel and stained with ethidium bromide. A DNA digest (pBR322/BamHI/MvaI) was also run on the same gel as a DNA size marker (left lane).

Fig. 3. Southern blot of DNA extracted from CLs on day 3 of the cycle (Intact), CLs on day 3 of pregnancy (Pregnant), CLs on day 3 of the cycle after hypophysectomy on day 1 (Hypox) and CLs treated with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) after hypophysectomy (3 CLs each).

ments were multiples of 180 bp. This result showed that oligonucleosomal DNA fragmentation occurred in the CLs of the intact animals.

Fig. 4. DNA fragmentation ratio of CLs on day 3 of the cycle (Intact), CLs on day 3 of pregnancy (Pregnant), CLs on day 3 of the cycle after hypophysectomy on Day 1 (Hypox) and CLs treated with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) after hypophysectomy. Values represent means + SEM. The values without a common letter are significantly different ($P<0.05$).

Fig. 5. Haematoxylin and eosin staining of CLs on day 3 of the cycle (Intact), CLs on day 3 of pregnancy (Pregnant), CLs on day 3 of the cycle after hypophysectomy on day 1 (Hypox) and CLs treated with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) after hypophysectomy. Scale bar: 20 μ m. Signs of CL regression including influx of neutrophils (thick arrows) and presence of apoptotic luteal cells (arrow heads) were observed in the intact and Hypox groups.

DNA fragments extracted from three CLs of each treatment group were electrophoresed together on a gel (Fig. 3). Oligonucleosomal DNA ladder patterns were detected for each group. In

Fig. 6. Numbers of apoptotic cells (A) and neutrophils (B) in CLs on day 3 of the cycle (Intact), CLs on day 3 of pregnancy (Pregnant), CLs on day 3 of the cycle after hypophysectomy on day 1 (Hypox) and CLs treated with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) after hypophysectomy. Values represent means + SEM. The values without a common letter are significantly different ($P<0.05$).

the intact and Hypox groups, strong signals were detected. On the other hand, weak signals were detected in the pregnant, H+PRL and H+PRL+eCG groups.

The DNA fragmentation ratios determined by the fluorometric measurement method are shown in Fig. 4. The DNA fragmentation ratio of the intact group was high, and that of the pregnant group was low. The DNA fragmentation ratio of the Hypox group was the same as that of the intact group. The DNA fragmentation ratios of the H+PRL and H+PRL+eCG groups were comparable to the pregnant group.

In the histological analysis, characteristic morphological changes in luteal regression were observed in the CLs of the intact group (Fig. 5). The CLs showed infiltration with large numbers of neutrophils (Fig. 6), shrinkage of luteal cells, absence of blood vessels and formation of apoptotic bodies. In contrast, there were no signs of luteal regression in the pregnant group. The luteal cells contained large nuclei with large cytoplasm. Many blood vessels could be seen without neutrophils and apoptotic cells in the CLs of the pregnant group (Fig. 5). In the Hypox group, regressive signs (infiltration of neutrophils, shrinkage of luteal cells, absence of blood vessels and formation of apoptotic bodies) were observed (Fig. 5), but the number of neutrophils and apoptotic cell were smaller than those of intact CLs (Fig. 6). In the H+PRL and H+PRL+eCG groups, regressive signs were not observed (Fig. 5), and the numbers of neutrophils and apoptotic cell were at the same

Fig. 7. Plasma (A) and CL (B) concentrations of progesterone (P4) on day 3 of the cycle (Intact), day 3 of pregnancy (Pregnant), day 3 of the cycle after hypophysectomy on day 1 (Hypox) and with PRL (H+PRL) or PRL plus eCG (H+PRL+eCG) treatment after hypophysectomy. Values represent means + SEM. The values without a common letter are significantly different ($P < 0.05$).

as those of the pregnant group (Fig. 6).

Effects of hypophysectomy and PRL and eCG replacement after hypophysectomy on luteal function

The mean concentrations of plasma and CL P4 are shown in Fig. 7; the P4 concentration of a CL was calculated as (P4 content of the CL) / (weight of the CL). In the Hypox group, the plasma and luteal P4 concentrations were at the same low level observed in the intact group. In the H+PRL group, although the luteal P4 concentrations were significantly higher than those of the intact and Hypox groups, the plasma P4 concentrations were at the same level observed in the other two groups. On the other hand, in the H+PRL+eCG group, although the luteal P4 concentrations were at the same level observed in the H+PRL group, the plasma P4 concentrations were significantly higher than in all the other groups.

Discussion

The purpose of this study was to evaluate how pituitary hormones act on luteal maintenance and regression in the cyclic hamster, examining the index of apoptosis in addition to past ones. The process of luteal regression in the cyclic hamster has been studied in detail previously [5, 6]. In the CL of the cyclic hamster, infiltration of neutrophils and appearance of apoptotic luteal cells are observed at 0900 and 1500 h on day 3, respectively, and a DNA

ladder pattern is detected by electrophoresis in the afternoon on day 3 of the cycle [5]. In the present study, many apoptotic luteal cells and neutrophils (Fig. 5), definite oligonucleosomal DNA ladders (Figs. 2, 3) and high DNA fragmentation ratios (Fig. 4) were detected in the CLs of the intact group. These results indicate that structural luteal regression was occurring in the intact animals by at least 1500 h on day 3 of the cycle. The plasma and luteal P4 concentrations of the intact group were very low (Fig. 7), and these results indicate that functional luteal regression already occurred. The results of intact group in this study are in agreement with the previous results [4–6].

In the present study, treatment with PRL after Hypox on day 1 of the estrous cycle significantly decreased the DNA fragmentation ratio of the CL (Fig. 4) and increased the CL weight (Fig. 1) compared with the Hypox group. Combination of PRL and 2 IU eCG after Hypox also decreased the DNA fragmentation ratio (Fig. 4) to the level of the pregnant group, and the CL weight (Fig. 1) and number of apoptotic cells (Fig. 6) showed same tendency as seen in the DNA fragmentation ratio data. In the present study, however, a clear difference was not found between the effects of PRL treatment alone on luteal morphology and those of eCG administration in addition to PRL treatment. These results suggest that PRL, but not eCG, is a primary and main factor for structural maintenance of the CL. In the hamster during early pregnancy, the plasma PRL concentration and CL size increases daily; on the other hand, the PRL concentration and CL size do not change during days 1 to 3 of the estrous cycle [20]. This positive correlation between CL weight and the plasma PRL concentration supports our present conclusion that PRL is a primary and main factor for structural maintenance of the CL in the hamster.

In the present study, treatment with PRL only increased the CL P4 concentrations but not the plasma P4 concentrations (Fig. 7), and PRL plus eCG treatment increased both the plasma and CL P4 concentrations to the pregnant level. These results suggest that PRL has stimulative effects on luteal P4 production that are insufficient for maintenance of the peripheral P4 concentrations and that gonadotropin, in addition to PRL, is necessary to maintain the peripheral P4 concentrations. This finding is in agreement with a past report [10]. Greenwald [9] reported that the combination of PRL and FSH is the minimal luteotropic complex in the hamster. After Hypox, daily administration of 1 mg PRL and 200 μ g FSH maintains pregnancy. Furthermore, LH is also necessary for optimal luteal function in the pregnant hamster. A small dose of a long-acting formed LH enhances the function of the minimal luteotropic complex in the pregnant hamster [21]. In the hypophysectomized pregnant hamster, the combination of PRL and eCG is the optimal treatment for maintenance of luteal function because eCG has strong FSH and weak LH function in the rodent ovary [10]. The concept of the luteotropic complex is different from that in the rat and is a feature of the hamster because PRL is the primary factor in the rat for maintenance of sufficient progesterone secretion from newly formed CLs [1, 2].

Based on the present results for the luteal and plasma P4 concentrations, it is possible that PRL and eCG have an effect on P4 secretion from the CL into the peripheral blood. Although there were no histological differences between the CLs of the PRL-only

treatment group and PRL plus eCG treatment group in the present study, it is possible that eCG affected luteal blood flow, vascularity or vascular permeability and resulted in an increased plasma P4 concentration. Greenwald reported that the size and vascularity of the CL is enhanced in hypophysectomized hamsters when small doses of LH are given along with the luteotropic complex of PRL and FSH [9], and FSH or LH administration increases ovarian steroid hormone secretion without blood flow change in the cyclic hamster [22].

The present results show that the effects of pituitary hormones on the CL in the estrous cycle are luteotropic and support the concept that luteal regression during the estrous cycle is due to the lack of luteotropic stimulation rather than to the direct effect of a luteolytic factor in the hamster. In the rat, PRL has dual luteotropic and luteolytic roles on the CL [23]. During early pregnancy, PRL acts as a primary luteotropic hormone, and acts as a primary luteolytic hormone for the CL in the cyclic rat [1–3]. Although PRL maintains both luteal function and structure in the pregnant rat [1], PRL maintains only luteal structure, but not luteal function, in the pregnant hamster [9]. This characteristic of the hamster is also not found in the rat.

Recently, the initiating and mediating mechanisms of apoptosis have been investigated in several species [3]. However, only a limited number of reports are available concerning this in the hamster. McCormack *et al.* [5] and Gaytan *et al.* [6] analyzed the luteal regression process involved in apoptosis in the cyclic hamster. They demonstrated that neutrophils appear in the CL before apoptosis and suggested the possibility that influx of neutrophils initiates luteal apoptosis. Chen *et al.* [24] reported that PRL withdrawal with bromocriptine influences Fas and Fas ligand expression, and to our knowledge this is the only report concerning study of the regulation of luteal apoptosis at the molecular level in the hamster. The present results suggest the possibility that PRL suppresses luteal apoptosis in the cyclic hamster, but further investigations are required to elucidate the control mechanisms of apoptosis during the estrous cycle in the hamster.

In the present study, the CL weights in the Hypox group were less than those in the intact group, and the numbers of neutrophils and apoptotic cells were less in the Hypox group than in the intact group. These results seem contradictory in terms of the process of structural luteal regression in the hamster, since infiltration of neutrophils and appearance of apoptotic cells occurs before CL weight reduction in the structural luteal regression of the hamster [5]. We do not know the reason for this at present.

In conclusion, the present results suggest that PRL restrains luteal apoptosis and maintains luteal structure and that the combination of PRL and eCG maintains the same luteal function in the cyclic hamster as seen in the pregnant hamster.

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