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Corpus Luteum Response to 6-chloro Δ^6-17 acetoxyprogesterone and HCG in the Cow

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An *in vivo–in vitro* incubation system was used to evaluate the effects of 6-chloro Δ^6-17 acetoxyprogesterone (CAP) and HCG on bovine corpus luteum (CL) growth and progesterone synthesis. Corpora lutea were removed from 15 heifers in three consecutive estrous cycles. Progesterone concentration and CL size were not affected by repeated CL removal. Variations in progesterone values for CLs within animals were similar to that between animals. Weight of corpora lutea formed after a CAP-synchronized estrus was equal to controls while progesterone concentration was increased. HCG, administered daily from Day 2 to Day 7 of the estrous cycle significantly ($p < 0.01$) increased CL size; however, these CLs were less responsive to *in vitro* HCG stimulation than control or CAP CLs. HCG given in single injections (1000 IU) on either Day 1 or 3 of an estrous cycle also significantly increased CL size. Progesterone concentration was not markedly altered in the larger CLs, but the total content of progesterone was significantly increased.

The use of synthetic progestins for estrous synchronization in cattle has been accompanied with lowered fertility at the first breeding (Wagner *et al.*, 1968, and Zimbelman, 1963). The exact cause of this impaired conception rate is not known; however, an unbalanced estrogen–progesterone ratio may be a factor. Lingering amounts of progestins may contribute to this imbalance as well as interfere with the functional status of a developing corpus luteum (CL) by inhibiting the release of luteotrophic hormone (LH). Additional unknown factors are very likely playing a role in this lowered pregnancy rate.

Hansel and Seifart (1967) summarized the evidence showing that LH is the primary luteotrophin in the cow. Donaldson and Hansel (1965) demonstrated that human chorionic gonadotropin (HCG) has luteotrophic properties similar to LH *in vivo*. HCG also duplicates the action of LH *in vitro* by stimulating progesterone synthesis in CL slices (Mason *et al.*, 1962). Therefore, if prolonged progestin administration results in impaired CL function, it may be possible to regain CL function by administering LH or a gonadotropin such as HCG at the proper time.

The technique of *in vitro* CL incubation as reported by Suarez Soto and Demare (1960) and further developed by Mason *et al.* (1962) and Armstrong *et al.* (1964) provides a ready tool for testing the effects of progestins and gonadotropins given *in vivo* on corpus luteum function.

The objective of the studies reported was to determine the effects of 6-chloro Δ^6-17 acetoxyprogesterone (CAP) and HCG on corpus luteum weight and *in vitro* progesterone synthesis in heifers undergoing normal estrous cycles.

MATERIALS AND METHODS

Experiment Design

Experiment 1. In the first experiment CLs were collected from 15 Hereford heifers (weighing 317–398 kg.) on Days 9, 10, or 11 of three successive normal estrous cycles. The CLs were weighed, sliced, incubated, and assayed for progesterone. A normal estrous cycle following each CL removal was achieved by allowing the heifer to exhibit a postremoval estrus followed by an estrous cycle of 18 to 22 days in length.

A total of 41 CLs were collected, and the data relative to various parameters of function were analyzed for within and between heifer variation. This supplied information as to whether an animal should provide its own control CL in future experiments.

Experiment 2. Pretreatment control CLs were removed from 27 Hereford heifers on Days 9, 10, or 11 of a normal estrous cycle. All heifers were allowed to exhibit a normal estrous cycle and were then assigned to three hormonal treatments so that all stages of the estrous cycle were equally represented in the treatment groups. One additional heifer was added to each of the CAP treatment groups to complete the 2 × 2 factorial arrangement as follows: 1) Pretreatment controls—27 heifers, 2) HCG treated—9 heifers, 3) CAP synchronized—10 heifers, and 4) CAP synchronized plus HCG treated—10 heifers.

Group 2 heifers received six daily subcutaneous injections of HCG[1] (1000 IU/day) from Day 2–Day 7 following a normal estrus (day of estrus = Day 0).

Group 3 heifers were treated orally with 10 mg CAP/day for 14 days followed by 5 mg CAP/day for 4 days.

Group 4 heifers were treated with CAP in the same manner as group 3 and in addition received 6 daily sc injections of HCG on Days 2–7 following the CAP synchronized estrus. In all groups the corpora lutea were removed on the 9th, 10th, or 11th day following either the CAP-synchronized or normal estrus. These CLs were weighed, sliced, incubated, and assayed for progesterone.

Thirteen of the heifers described above were used again to provide posttreatment CLs. Following the removal of a treatment CL, the heifers were allowed to exhibit a normal estrous cycle, and the corpus luteum was removed on Day 10 of the subsequent cycle. These CLs were then compared with the pretreatment corpora lutea collected from the same heifers.

Experiment 3. The effect of HCG given as a single injection on corpus luteum function was investigated in experiments 3 and 4. In Exp. 3, the CLs from 11 normal cycling heifers were evaluated (negative control) while 45 heifers were synchronized with CAP. The HCG treatments were given on the third day after the CAP-synchronized estrus as follows: 1) Control (11 heifers), 2) CAP—no HCG (9 heifers), 3) CAP + 1000 IU HCG (15 heifers), 4) CAP + 2000 IU HCG (13 heifers), and 5) CAP + 4000 IU HCG (8 heifers).

[1] HCG—Human chorionic gonadotropin purchased from Ferring AB, Malmo, Sweden. Assayed against the 2nd International Standard for chorionic gonadotropin, Division of Biological Sciences, National Institute for Medical Research, Mill Hill, London, England.

The methods of injecting HCG, CL removal, and incubations were carried out as in Exp. 2.

Experiment 4. Fourteen heifers were synchronized with CAP, and HCG was injected approximately 12 hr after the heifers were observed in estrus. The animals were allotted to treatments as follows: 1) No HCG (6 heifers), 2) 1000 IU HCG (4 heifers), and 3) 2000 IU HCG (4 heifers).

The HCG was given sc and the CLs were removed on Day 10 and weighed. *In vitro* incubations were not performed on the CL tissue in this experiment.

Tissue Preparation and Progesterone Analyses

All corpora lutea evaluated in these studies were obtained by manual expression from the ovary via a paralumbar incision. After being trimmed of excess connective tissue, the CLs were halved and placed on chilled saline saturated filter paper and weighed. Tissue slices for *in vitro* incubations were prepared in the cold (5C) as described by Mason *et al.* (1962). CL slices were randomly distributed in aliquot samples, weighed, and placed in incubation flasks containing 5 ml Krebs–Ringer bicarbonate buffer (KRB pH approximately 7.4). *In vitro* incubation treatments were carried out in duplicate in a Dubnoff metabolic incubator as described by Mason *et al.* (1962) and immediately frozen at −20C. The *in vitro* treatments were as follows: 1) Unincubated controls, 2) Incubated controls, and 3) Incubated plus HCG. The desired level of HCG in the incubation medium was contained in 5 ml of KRB in Exp. 1. In all other experiments only 1.0 IU HCG was used to evaluate *in vitro* CL progesterone synthesis.

Progesterone Analysis

[4-^{14}C]progesterone (0.009 μCi) was added to each sample. Following thawing at room temperature the tissue and incubation media together were homogenized in a Thomas tissue grinder. Diethyl ether extraction and two dimensional thin-layer chromatography (TLC) was performed according to Armstrong *et al.* (1964) with a modification of solvent systems from a 5:2 ratio to a 2:1 ratio. This resulted in an increased mobility of the progestins on silica gel H. The progestin areas were located under an ultraviolet (UV) light, scraped, and eluted with methanol. UV absorbance was measured at wave lengths of 230, 240, and 250 nm and quantitated by using Allen's (1950) formula. Recovered [^{14}C]progesterone was determined in a Packard Tri-carb liquid scintillation spectrometer, and all progesterone values were corrected to 100%. Progesterone values are reported as micrograms in tissue and medium divided by grams of tissue incubated (μg/gm). All [^{14}C]progesterone recovery percentages ranged from 75–100%.

When sufficient 4 pregnen-20β-ol-3-one (20β-ol)

was present, this steroid was quantitated. However, in most cases 20β-ol was not detected in nonincubated tissue and in incubated tissues it was quite constant, representing 10–20% of the progesterone values. Therefore, the levels of 20β-ol were not included in the analysis of these data.

In order to evaluate sources of variation in the data the effects of the following variables were analyzed by appropriate statistical procedures: 1) Progesterone concentration treatment effects—analysis of variance of log transformed data; 2) Tissue sample size effect on *in vitro* progesterone synthesis—covariance; 3) The effect of removing CLs on Days 9, 10, or 11 of the estrous cycle—analysis of variance; and 4) The presence and size of a fluid filled cavity on the progesterone concentration of a CL—covariance. The variation contributed by factors 2–4 was nondetectable with each having a calculated *F* ratio of less than 1.0.

RESULTS AND DISCUSSION

Experiment 1. In order to establish an *in vitro* dose of HCG which would have some discrimination in stimulating progesterone synthesis rather than result in maximum stimulation regardless of the functional state of the CL tissue, four levels of HCG were tested. A summary of the *in vitro* HCG dose level response on CL progesterone synthesis is shown in Table 1. One IU of HCG added to the incubating CL tissue resulted in a less than maximal stimulation of progesterone synthesis with a higher degree of repeatability. Therefore, 1.0 IU of HCG was selected for use in all future experiments to test CL function. Additionally, it was found that 1.0 IU of HCG stimulated progesterone similarly to 0.01–0.05 μg of NIH-LH-S7 (Table 1). Moody and Hansel (1969) have reported that minced luteal tissue had 146 μg progesterone/g following stimulation with 0.016 μg LH.

TABLE 1

EFFECT OF HCG AND LH *In Vitro* ON CL PROGESTERONE SYNTHESIS

HCG (IU/flask[a])	Hormone level				
	0	0.1	0.4	1.0	10.0
μg/g[c]	94 (41)[b]	111 (11)	131 (15)	168 (41)	200 (16)
SE	2.97	4.65	9.64	6.30	13.16
LH (μg/flask[a])	Hormone level				
	0	0.01	0.05	0.1	0.5
μg/g	107 (6)	155 (6)	183 (3)	220 (3)	215 (3)

[a] *In vitro* incubations were prepared so that 5 ml KRB contained the dose of hormone

[b] No. of observations

[c] mcg/g = total progesterone in flask ÷ tissue weight

The effect of repeated corpus luteum removal on individual CL size and progesterone synthesis was determined. Corpus luteum weights were not altered by repeated removal (Table 2). The increase in progesterone concentration following *in vitro* incubation approximated 3-fold. This response was constant for all three extirpations (Table 2). When 1.0 IU of HCG was added to the incubation medium, the progesterone concentration was increased 5-fold (Table 2).

In order to determine if variation could be removed by having an animal provide a control CL prior to an *in vivo* CL function experiment the data were analyzed for sources of variation. The variance of progesterone concentration was proportional to the mean concentration, i.e., as the progesterone concentration increased, the variance among the replications increased proportionally. This observation clearly indicated that the data be transformed to logarithms for the analysis of variance which is presented in Table 3. The variation attributed to the CLs from different animals within the same extirpation was equal to the variation between CL removals from the same animal. The variances between animals and within an animal indicated that paired data (each animal acting as its own control) were not needed.

Experiment 2. When 1000 IU HCG was injected sc for 6 days, a marked increase in

TABLE 2

EFFECT OF CL EXTIRPATION ON VARIOUS PARAMETERS OF THE CORPUS LUTEUM (Exp. 1)

CL[a] removal	No. animals	CL wt g	Noninc.[b] conc. μg/g	Inc.[c] conc. μg/g	*de novo*	Inc.[d] HCG stim. conc. μg/g	*de novo*	Total noninc. content μg
1	14	4.64	33	102	69	179	77	147
2	13	4.45	28	92	64	163	71	124
3	14	4.54	31	93	62	164	71	139
Total	41	4.55	30.3	95	65	166	71	137.4

[a] All corpora lutea were surgically removed on Days 9, 10, or 11 of a normal estrous cycle.

[b] Nonincubated—all samples sliced, weighed, and frozen.

[c] Incubated—All samples gassed with 95% O_2:5% CO_2 in Erlenmeyer flasks and incubated for 2 hr in 5 ml Krebs-Ringer Bicarbonate buffer at 37C.

[d] Incubated-stimulated—All samples treated similar to inc. with 1.0 IU HCG added to the incubation media.

TABLE 3

ANALYSIS OF VARIANCE OF LOG$_e$ TRANSFORMED PROGESTERONE CONCENTRATION (μg/g)

Source	DF	MS
In vitro tissue treatment	2	61.932*
Between animals	13	0.307
Between CL extirpations	2	0.372
Between extirpation within animal	27	0.321
Between animal within extirpation	38	0.311
Remainder	198	0.046

* $p < 0.01$.

CL weight occurred as seen in Table 4. This increase in CL size was similar for normal and CAP-synchronized heifers (Groups 2 and 4, Table 4). CAP treatment had no effect on CL size (Group 3 *vs.* Group 1) while concentration (μg/g) of progesterone was significantly increased ($p < 0.01$). The rate of *in vitro* progesterone synthesis was accelerated in CLs from heifers treated with CAP ($p < 0.01$) as seen by the average *de novo* production of 108 μg/g for Groups 3 and 4 while Groups 1 and 2 produced an average of 82 μg/g during incubation (Table

TABLE 4

EFFECT OF CAP AND HCG *In Vivo* ON BOVINE CL FUNCTION *In Vitro* (Exp. 2)

Group	Treatment[1]	CLs No.	CL WT[2] g	Progesterone Concentration					
					Incubated		Inc. Stim.		
				Non-inc.[2] μg/g	Conc.[2] μg/g	*de novo* Synthesis[2] μg/g	Conc.[2] μg/g	*de novo* Synthesis[2] μg/g	Content[2] μg/CL
1	Control	27	4.33[a]	28[a]	108[a]	80[a]	186[a]	78[c]	122[a]
2	HCG	9	8.92[b]	28[a]	112[a]	84[a]	168[a]	56[d]	243[b]
3	CAP	10	4.31[a]	35[b]	138[b]	103[b]	201[b]	63[c]	146[a]
4	CAP plus HCG	10	9.04[b]	40[b]	154[b]	114[b]	209[b]	55[d]	361[b]

[1] All CAP treated heifers received 10 mg CAP/head/day for 18 days. All HCG treated heifers received 1000 IU HCG/head/day for six days from Day 2 to Day 7.

[2] Values having superscripts a and b are different ($p < 0.01$). (Duncan. 1955)

[3] Values having superscripts c and d are different ($p < 0.10$). (Duncan, 1955)

4). When 1.0 IU HCG was added to the incubation medium, the *de novo* progesterone synthesis rate of CL tissues treated with HCG was not stimulated to the same degree as the control and CAP tissue ($p < 0.01$) (Table 4). This would indicate that the *in vivo* HCG treatments resulted in some degree of stimulation that caused the tissue to be less responsive to *in vitro* HCG stimulation. Moody and Hansel (1967) observed a similar phenomenon in CLs from *in vivo* HCG treated dairy heifers. Moody and Hansel (1970) suggest that this may be due to a depletion of essential precursors. The *in vivo* HCG treated groups contained significantly greater progesterone within CL than the control groups 1 and 3, due primarily to the increased CL size. Schomberg and coworkers (1967) also demonstrated that HCG stimulates *in vivo* progesterone synthesis by measuring an increase in peripheral blood progesterone concentrations.

Corpora lutea from heifers in Groups 2–4 (Table 4) were evaluated during the normal estrous cycle following treatment as shown in Table 5. Statistical analysis showed an absence of carry-over effects from previous treatments; therefore, the data were combined into one posttreatment control group. The pretreatment and posttreatment CLs were not different in any of the parameters measured (Table 5). Therefore, apparently there is no residual effect of a CAP or HCG treatment given during one estrous cycle on the CL formed during subsequent cycles.

Experiment 3. HCG, given on Day 3 of the cycle following CAP treatment produced an increase in CL weight similar to six daily injections (Tables 3 and 6). A single dose of 1000 IU HCG resulted in an increase ($p < 0.01$) in CL size. Doses of 2000 IU and 4000 IU produced additional growth responses (Table 6). As observed in Exp. 2, CLs from CAP-treated heifers had a slightly increased rate of *de novo* progesterone synthesis. However, the *de novo* progesterone synthesis following incubation and incubation–stimulation in CL tissue from animals which received 1000 and 2000 IU HCG fell below that of the controls and CAP-treated heifers. The *in vitro* response of 4000 IU HCG treated CL tissue was dissimilar to the two previous HCG levels in that an increase in initial progesterone concentration and *de novo* synthesis was observed. It is of significance that in the analysis of variance of the data from Exps. 2 and 3 the error variance (animals within treatment) was almost identical to the between animal and within animal variance from Experiment 1 (Table 3). These error variances were 0.356 and 0.561 for Exps. 2 and 3, respectively. This further demonstrates the validity of using these procedures for evaluating bovine CL function.

Further evaluation of the effect of HCG on CL growth was undertaken in Exp. 4. HCG (1000 and 2000 IU) given on day 0

TABLE 5

COMPARISON OF PRE- AND POSTTREATMENT CL FUNCTION (Exp. 2)

Group	No.	Ave CL Wt (g)	Progesterone Concentration			Total progesterone content μg
			Noninc. μg/g	Inc. μg/g	Inc.–stim. μg/g	
Pretreatment[a]	13	4.02	28	105	149	104
Posttreatment[b]	13	4.14	26	107	178	105

[a] CLs were removed following a normal estrus.

[b] CLs were removed following a normal estrus subsequent to the treated cycles shown on Table 4.

TABLE 6
EFFECT OF CAP AND HCG (SINGLE DOSE) ON CL FUNCTION (Exp. 3)

Treatment[1]	CLs No.	CL Wt (g)	Noninc. μg/g	Incubated		Incubated–stim.		CL Content μg/CL
				μg/g	*de novo*	μg/g	*de novo*	
Control	11	5.0[a]	37[a]	121[a]	84	209[b]	88	188[a]
CAP	9	5.2[a]	38[a]	133[a]	95	227[b]	94	189[a]
CAP + HCG 1,000	15	8.6[b]	41[a]	120[a]	79	188[a]	68	363[b]
CAP + HCG 2,000	13	9.6[bc]	40[a]	116[a]	76	184[a]	68	391[b]
CAP + HCG 4,000	8	10.6[c]	50[b]	156[b]	106	235[b]	79	517[c]

[1] All heifers were treated with CAP (10 mg/head/day) for 18 days; HCG was given subcutaneously three days after the day of estrus. Values having the same superscript are equal ($p < 0.05$). (Duncan, 1955)

(12 hr after the onset of estrus) again resulted in a significant ($p < 0.05$) increase in the CL size (Table 7). However, the degree of CL growth appears diminished when the HCG was given at Day 0 as compared with the Day 3 treatment (Table 6 *vs.* 7). Even though the CL growth response was lessened when HCG was given at Day 0, the significant increase that occurred demonstrates that the endogenous source of progesterone can be increased. Armstrong and Black (1966) concluded that the CL size is the most important factor determining endogenous progesterone supplies. Therefore, increasing the CL size affords a technique by which the endogenous estrogen-progesterone ratio can possibly be altered.

TABLE 7
EFFECT OF HCG ON CORPUS LUTEUM SIZE IN CAP-SYNCHRONIZED[a] BEEF HEIFERS (Exp. 4)

Amount of HCG[b]	No. of heifers	Average CL weight g*
0	6	3.6
1000 IU HCG	4	4.7
2000 IU HCG	4	6.0

[a] All heifers were treated with CAP to synchronize estrus.

[b] HCG was administered subcutaneously approximately 12 hr after a heifer was observed in estrus.

* Means different from one another ($p < 0.05$).

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