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Identification and measurement of collagen in the bovine corpus luteum and its relationship with ascorbic acid and tissue development

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A preliminary quantitative and qualitative analysis of the collagen component of the bovine luteal extracellular matrix is reported. The amount of collagen present in corpora lutea from four stages of luteal development was estimated from measurements of hydroxyproline. The tissue content of ascorbic acid, a cofactor in collagen biosynthesis, was also determined. Extracts of luteal tissue were subjected to polyacrylamide gel electrophoresis and compared with patterns derived from standard preparations of commonly occurring collagens. Measurements of hydroxyproline showed that collagen contributed up to about one-sixth of the luteal dry matter (3% of wet weight), with the highest absolute amounts occurring in mature tissue. Ascorbic acid was present in tissue from all stages of the luteal phase, with the highest concentrations occurring in the midluteal phase. Hydroxyproline content was closely correlated with tissue weight and ascorbic acid content during the first stage of the luteal phase, indicating that collagen is produced as a component of tissue growth, supported by the concurrent accumulation of its biosynthetic cofactor. The highest concentrations of hydroxyproline occurred in the final stage of the luteal phase, suggesting that luteolysis is associated with a preferential loss of cellular rather than extracellular material. From the electrophoretic analysis, the fibrillar collagen type I could be identified at all stages of the luteal phase. We conclude that collagen is a major component of the luteal extracellular matrix and is produced as an integral component of tissue development. The presence of a fibrillar rather than a basement membrane type of collagen suggests a significant change in gene expression when follicular tissue is remodelled into that of the corpus luteum.

Introduction

The corpus luteum develops from the tissues of the ovulating follicle by remodelling and growth. At the same time, differentiation of endocrine cells results in the transition from the follicular to the luteal phase. A number of studies have described the events in the tissue that lead to the rupture of the ovulating follicle (see Bjersing and Cajander, 1974a–c; Espey, 1980, 1991), including the release of proteases that destroy the collagen component of the follicle wall. The mechanism of tissue remodelling by which the corpus luteum is formed is less well described. In particular, very little is known about the components of the luteal extracellular matrix or their cellular origins. Similarly, little is understood about the time relationship between luteal development, the differentiation of luteal cells and the appearance of extracellular structures.

A morphometric analysis of bovine tissue indicated that extracellular space comprised 30% of the cross-sectional area of the corpus luteum on day 6 of the cycle and 15% on day 15 (Parry *et al.*, 1980). In perfusion-fixed rat tissue, intercellular space accounted for 5% of the area (Meyer, 1991). The nature of this material has not been described and the assumption must therefore be that it consists of conventional extracellular components including collagen (Labat-Robert *et al.*, 1990). Luteal cells lack a basal lamina, except where they are in close apposition to capillaries (Meyer, 1991), and it is therefore expected that any collagen present in the extracellular matrix will be of the fibrillar (interstitial) rather than the basement membrane type (van der Rest and Garrone, 1991). Fibrillar collagens, although present in the ovarian capsule and stroma, are absent from the tissues of the follicle (Kaneko *et al.*, 1984; Palotie *et al.*, 1984; Christiane *et al.*, 1988). The follicular basement membrane, separating the granulosa and thecal layers, contains collagen type IV in addition to laminin, fibronectin and heparan sulfate proteoglycan (Bagavandoss *et al.*, 1983; Yoshimura *et al.*, 1991; Leardkamolkarn and Abrahamson, 1992).

The aim of the present study was to establish some basic information about the luteal extracellular matrix, in particular about the collagen component and its relationship to luteal development. Assays of hydroxyproline, a collagen-specific amino acid, were performed on luteal tissue from different stages of the cycle to estimate the quantity of collagen likely to be present. Assays for ascorbic acid were also performed since this is an essential cofactor in the expression and synthesis of collagen (Pinnell, 1985; Bornstein and Sage, 1989; Chojkier *et al.*, 1989; Padh, 1991). Ascorbic acid is abundant in the corpus

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luteum (Sheldrick and Flint, 1989) and should therefore be present during periods of collagen synthesis (Espey and Coons, 1976). Finally we made a preliminary electrophoretic analysis of luteal proteins to identify the type of collagen present.

Materials and Methods

Tissue

Ovaries bearing corpora lutea, from cows of unknown reproductive status, were obtained within 1 h of slaughter from a local abattoir and transported to the laboratory on ice. After rinsing in saline, corpora lutea were excised, taking particular care to remove all capsular tissue. They were assigned to four groups, according to the morphological criteria (colour, vascularization, epithelial covering) of Ireland *et al.* (1980), representing the following approximate periods of the luteal phase: Stage I – days 1–4, Stage II – days 5–10, Stage III – days 11–17 and Stage IV – days 18–20. Each corpus luteum was sectioned, blotted to remove any fluid, weighed and assayed for ascorbic acid and hydroxyproline. Samples from other corpora lutea were weighed and dried to constant weight to determine the proportions of wet and dry matter. Representative tissue was also retained for collagen extraction and electrophoresis. Reagents were of analytical grade, from FSA Laboratory Supplies (Loughborough) or Sigma (Poole), unless otherwise stated.

Measurement of hydroxyproline

Hydroxyproline was determined by a modification of the Chloramine T method of Edwards and O'Brien (1980). Weighed tissue samples were homogenized in HCl (6 mol l^{-1}, 10 mg tissue ml^{-1}) and hydrolysed in a pressure cooker for 2 h. After cooling, samples were neutralized with concentrated NaOH, lyophilized to dryness and taken up in 3 ml assay buffer (0.16 ml citric acid l^{-1}, 0.8% v/v glacial acetic acid, 1 mol sodium acetate l^{-1}, 0.57 mol NaOH l^{-1}, 20% v/v *n*-propanol, pH 6.0–6.5). Aliquots of 1 ml were taken for assay: these were diluted with 2 ml assay buffer and reacted with 1.5 ml freshly prepared 50 mmol chloramine T solution l^{-1} for 20 min. A colour reaction was produced by addition of 1.5 ml aldehyde–perchloric acid reagent (1.0 mol *p*-dimethyl-amino-benzaldehyde l^{-1}, 60% v/v *n*-propanol, 18% v/v perchloric acid) for 15 min at 60°C with gentle shaking. Absorbance at 550 nm was measured within 3 h. Working standards of trans-4-hydroxy-L-proline, over the range 7.65–121 μmol l^{-1}, were prepared in assay buffer. The intra-assay coefficient of variation was 8% and the minimum detectable concentration was 1.25 μmol l^{-1}.

Measurement of ascorbic acid

Ascorbic acid was assayed by the colorimetric method of Smith (1972). Colour reagent was prepared as follows: 2 ml of an equivolumetric mixture of glacial acetic acid and 10% sulfuric acid, containing 0.2% w/v (12 mmol l^{-1}) 2-nitro-4-methoxyaniline (4-amino-3-nitroanisole; ICN Biomedicals, High Wycombe) was added to an equal volume of 30 mmol sodium nitrite l^{-1}, mixed until the orange colour disappeared and stabilized by addition of 75 ml absolute ethanol. Samples of freshly excised tissue were weighed and homogenized in freshly-prepared, ice-cold metaphosphoric acid (3% w/v, containing 2.5 mmol EDTA l^{-1}). Standard solutions of ascorbic acid (Sigma) were prepared in the same solution. Colour reagent (650 μl) was added to each sample/standard (100 μl), mixed and incubated at room temperature for 15 min followed by addition of 250 μl NaOH (2.5 mol l^{-1}). After further mixing to develop the blue colour, absorbance at 670 nm was determined. Absorbance was stable for up to 18 h at room temperature. The standard curve produced a straight line between 25 and 500 μmol ascorbic acid l^{-1}, with a minimum detectable concentration of 15 μmol l^{-1}. The intra-assay coefficient of variation was 6.3%.

Table 1. Comparison of percentage wet matter in samples of bovine corpus luteum, representing the early (Stage I, Ireland *et al.*, 1980), mid-(Stages II and III) and late (Stage IV) luteal phase, estimated by drying to constant weight

Period	Number of samples	Mean wet matter (%)	SEM
Early	7	84.01^{AB}	0.58
Middle	7	80.64^{A}	0.73
Late	7	79.27^{B}	0.43

^{AB}Values with a common superscript are significantly different ($P < 0.001$; ANOVA and least significant difference test).

Collagen extraction

Collagen was extracted qualitatively by the salt precipitation procedure of Miller and Rhodes (1982). Tissue was homogenized in washing buffer (10 ml (100 mg)$^{-1}$ tissue) containing 4.5 mol NaCl l^{-1}, 50 mmol Tris l^{-1}, 20 mmol EDTA l^{-1}, 1 mmol diisopropyl fluorophosphate (DFP) l^{-1}, 2 mmol *N*-ethylmaleimide (NEM) l^{-1}, pH 7.5, and centrifuged at 12 000 ***g*** for 15 min. The pellet was resuspended in buffer, recentrifuged and then washed in distilled water (containing EDTA, DFP and NEM, as above) to remove the salt. The pellet was resuspended in extraction buffer (0.5 mol acetic acid l^{-1}, with EDTA, DFP and NEM as above) and stirred vigorously overnight. After filtration (nylon mesh) to remove large, insoluble residues, the preparation was centrifuged (for 1 h at 47 000 ***g***). Crystalline NaCl was added to the supernatant to 2 mol l^{-1}, and collagen allowed to precipitate overnight. A pellet was obtained by further ultracentrifugation (for 1 h at 47 000 ***g***) and suspended in 0.5 mol acetic acid l^{-1}. After dialysis against acetic acid to remove salt, the preparation was lyophilized and stored at −20°C until electrophoresis.

Electrophoresis

Collagen preparations were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970; reagents from Biorad

Table 2. Analysis of bovine corpora lutea for fresh weight and the concentrations of ascorbic acid, hydroxyproline and collagen

		Fresh weight (g)			Ascorbic acid (μmol g^{-1})			Hydroxyproline (μmol g^{-1})			Collagen (mg g^{-1})
Stage*	*n*	Mean	SEM	Range	Mean	SEM	Range	Mean	SEM	Range	Range
I	13	1.70^{AB}	0.43	0.33–5.48	3.39^{Aa}	0.46	1.49–7.01	6.33^{A}	0.69	1.60–10.60	1.70–11.12
II	10	5.76^{Aa}	0.50	2.93–7.83	8.51^{a}	0.34	6.84–10.69	9.46^{a}	1.14	5.57–18.31	5.81–19.17
III	12	6.88^{BC}	0.45	3.84–9.21	10.34^{AB}	0.46	7.30–12.18	9.76^{b}	0.91	0.92–14.95	0.99–15.66
IV	18	3.06^{Ca}	0.47	0.38–6.83	4.94^{B}	1.15	0.29–16.44	18.00^{Aab}	1.75	7.70–31.58	8.05–33.10

*Classified according to Ireland *et al.* (1980). Collagen concentrations were calculated by assuming that hydroxyproline comprises 12.5% by weight of collagen (Edwards and O'Brien, 1980; Hay, 1981). Figures in a column with a common superscript were significantly different (ANOVA, Tukey's test): Uppercase: $P < 0.001$; lowercase: $P < 0.01$.

Table 3. Regression analysis of the relationship between the absolute hydroxyproline content of bovine corpora lutea and other parameters (fresh weight, ascorbic acid concentration per unit fresh weight, absolute ascorbic acid content) at four stages of the luteal phase*

		Fresh weight		Ascorbic acid			
				Concentration		Absolute	
Stage (s)	*n*	r^2	*P*	r^2	*P*	r^2	*P*
I–IV	53	0.54	<0.001	0.29	<0.001	0.42	<0.001
I	13	0.94	<0.001	0.19	NS	0.74	<0.001
II	10	0.29	NS	0.01	NS	0.46	<0.05
III	12	0.05	NS	0.00	NS	0.04	NS
IV	18	0.48	<0.01	0.21	NS	0.18	NS
I–II	23	0.75	<0.001	0.49	<0.001	0.75	<0.001
III–IV	30	0.36	<0.001	0.21	<0.01	0.25	<0.01

*Classified according to Ireland *et al.*, 1980. NS: not significant.

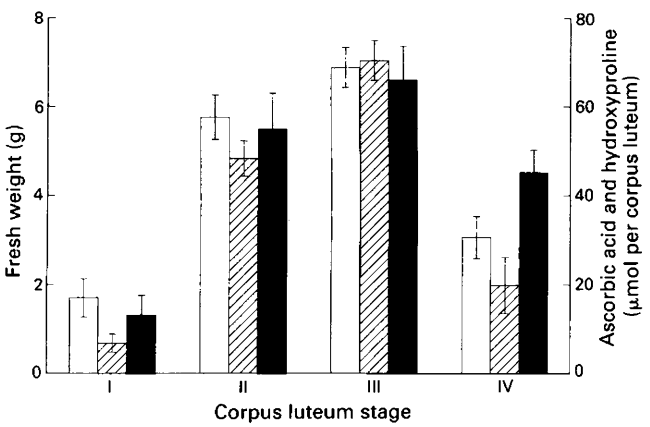

Fig. 1. Mean (±SEM) (□) fresh weight, and (▨) ascorbic acid and (■) hydroxyproline contents of bovine corpora lutea at Stages I ($n = 13$), II ($n = 10$), III ($n = 12$) and IV ($n = 18$) of the luteal phase.

Laboratories, Hemel Hempstead) under non-reducing conditions using a 6% gel at 120 V. Sample and standard collagen preparations were dissolved in 12 mmol HCl l^{-1} and taken up in loading buffer (0.3 mol Tris l^{-1}, 5% v/v SDS, 50% glycerol, with bromophenol blue). Approximately 5 μg protein was applied to each lane. The following commercial preparations of frequently occurring collagens were used as standards to provide peptide maps (Miller and Rhodes, 1982; Lillie *et al.*, 1987): collagen type I (Sigma, Type III from calf skin), collagen type III (Sigma, Type X from human placenta), collagen type IV (Sigma, Type IV from human placenta), collagen type V (Sigma, Type IX from human placenta).

Statistical analysis

Data were evaluated by ANOVA (CSS:Statistica) and differences between individual means were assessed by the least significant difference test or Tukey's test for unequal sample sizes, as appropriate. Because the normality of distribution of the sample data could not be assumed, the presence of significant variation indicated by ANOVA was confirmed using the nonparametric Kruskal–Wallis test.

Results

Estimates of the proportion of dry matter in luteal tissue ranged from 14 to 22%. Samples from the midluteal phase (Stages II

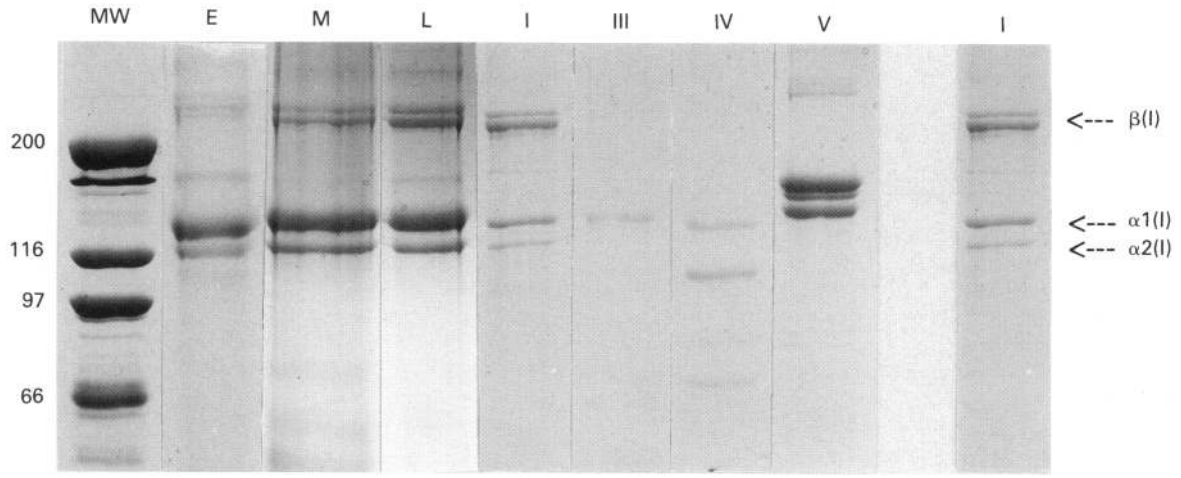

Fig. 2. SDS-PAGE of collagen extracted from early (E, Stage I, Ireland *et al.*, 1980), middle (M, Stages II and III) and late (L, Stage IV) phases of the luteal phase, in comparison with standard preparations (Sigma) of collagen types I, III, IV and V. The track for collagen type I is reproduced to the right to identify specific peptides (Lillie *et al.*, 1987; Miller and Rhodes, 1982). MW: molecular weight markers.

and III) were not significantly different from those from Stage IV (Table 1). Samples from Stage I had approximately 5% more wet material than those from later stages, which is consistent with the softer and serosanguineous nature of the earlier samples (Ireland *et al.*, 1980). In view of the small difference between the groups, concentrations of hydroxyproline and ascorbic acid were for convenience expressed as a fraction of fresh weight of tissue.

A total of 53 corpora lutea, at four stages of the luteal phase, were weighed and analysed for hydroxyproline and ascorbic acid (Table 2). Wide variations in weight were observed among the corpora lutea at all stages. Hydroxyproline concentrations varied least during Stage I, suggesting a close relationship between hydroxyproline production and tissue growth. This was confirmed by a correlation coefficient of 0.94 between total hydroxyproline content and tissue fresh weight during Stage I compared with 0.54 for the whole data set (Table 3). The highest concentrations of hydroxyproline occurred in Stage IV and were associated with a significant decline in weight of the corpus luteum as compared with Stage III. An estimate of collagen concentrations, based on the concentration of hydroxyproline and its known frequency in the collagen molecule (Edwards and O'Brien, 1980; Hay, 1981) suggested that collagen accounted for up to approximately 3% of the fresh weight of the tissue (equivalent to approximately 17% of the dry matter). Ascorbic acid was present in all corpora lutea but concentrations were highest during the midluteal phase (Stages II and III). The concentration fell markedly as tissue weight declined in Stage IV.

The mean absolute amounts of hydroxyproline and ascorbic acid present in the corpus luteum are given in comparison with the mean fresh weight (Fig. 1). A regression analysis was performed using ascorbic acid as a concentration and as an absolute amount, within and across the designated luteal stages to examine the relationship between ascorbic acid and the absolute amount of hydroxyproline in the tissue (Table 3). Over the complete data set, both ascorbate parameters were significantly correlated with hydroxyproline content, but the regression coefficient was higher for the absolute parameter. When divided into individual luteal stages, significant correlations with ascorbic acid concentration were lost. Significant correlations with the absolute amount of ascorbic acid remained for Stages I and II, and was particularly marked for Stage I. When the data were divided into early (Stages I and II) and late (Stages III and IV) phases, there was a strong correlation between hydroxyproline and ascorbic acid during the early phase.

Electrophoretic analysis of collagen extracted from early (Stage I), middle (Stages II and III) and late (Stage III) phases of the luteal phase revealed peptide patterns similar to that of collagen type I and unlike those of the other types of collagen examined. The result obtained on three to five occasions with corpora lutea of various sizes within each phase is shown (Fig. 2). The α_1(I), α_2(I) and β(I) peptide bands appeared at similar relative densities to those in the standard preparation in a pattern similar to that described by Miller and Rhodes (1982). Other unidentified bands were visible in the preparations from all corpora lutea as well as in the standard type I preparation.

Discussion

This study has, for the first time, identified collagen as a significant component of the bovine corpus luteum. On the basis of hydroxyproline estimation, collagen accounted for up to about one-sixth of the luteal dry matter. It therefore appears to be a major fraction of the abundant extracellular material present in luteal tissue (Parry *et al.*, 1980). Hydroxyproline is an amino acid found in collagen and a limited range of other proteins with collagen-like, trihelical domains (van der Rest and Garrone, 1991). Among the latter, it is likely that at least three would be present in the corpus luteum: the blood complement protein C1q and the macrophage scavenger receptor, which are presumably present in all vascularized tissues, and acetylcholinesterase (Luck, 1990). At this stage it is not possible to estimate the contribution of such proteins to the measured hydroxyproline, but it is expected to be relatively small. Neither complement C1q nor acetylcholinesterase appeared in the electrophoretic gel when added as pure preparations to the

collagen extraction procedure (data not shown). Notwithstanding this uncertainty, the electrophoretic analysis clearly identified collagen type I in tissue from all stages of the luteal phase and we therefore conclude that collagen is a major component of the luteal extracellular matrix.

The total amount of hydroxyproline in the corpus luteum was closely related to tissue weight, particularly during the first two stages of the luteal phase. As these stages represent the period of growth and maturation of the organ, the collagen is probably deposited as an integral component of tissue development. High concentrations were observed in the largest corpora lutea of Stages II and III, suggesting that collagen deposition continues beyond the period of most intense tissue growth. In the final stage of the corpus luteum (Stage IV), the concentration of hydroxyproline increased sharply as the tissue weight declined, with no significant change in the proportion of dry matter. This indicates that collagen is retained during luteolysis relative to the loss of other, presumably cellular, material.

The basis for examining ascorbic acid in this study was its established role as a cofactor in collagen biosynthesis. The concentrations we observed were of similar magnitude to those previously described in sheep corpora lutea (Sheldrick and Flint, 1989). The two studies also appear to show similar patterns of concentration over the luteal phase, with a rise during the development phase and a sharp decline during luteolysis. Although the relatively high uptake and storage of ascorbic acid by the corpus luteum is well recognized (Stansfield and Flint, 1967), its value to the organ was unclear. Ascorbic acid promotes the luteinization of granulosa cells (Luck and Jungclas, 1987, 1988) and enhances their response to stimulation (Luck, 1990; Luck and Münker, 1991), but the quantity available in the corpus luteum appears to exceed that required to support the biosynthesis of oxytocin and steroid hormones (Levine and Morita, 1985; Sheldrick and Flint, 1989). In the study reported here, there was a strong correlation between the amounts of ascorbic acid and hydroxyproline present in the corpus luteum, particularly during the developmental phase. This finding suggests that an important additional role for luteal ascorbic acid is the support of collagen synthesis during tissue formation and maturation.

The electrophoretic analysis identified collagen type I as an easily extractable component of luteal tissue. This result confirms an electron microscope observation (J. H. Payne, unpublished) of abundant fibrillar collagen surrounding the cells of the sheep corpus luteum. Although the presence of collagen type I in the present study was clear from its peptide map, this does not exclude the possibility that other collagens are also present. The extraction procedure used is a general one for all types of collagen and was perceived to be quantitatively somewhat inefficient. The range of purified types of collagen available for comparison was also limited. It is likely that other extraction procedures, more specifically suited to particular collagen types, or precise immunohistological techniques, would reveal other collagens in the same tissue. Indeed, not only the extracted samples but also the standard collagen type I preparation showed peptide bands other than those attributable to the α(I) and β(I) subunits.

The clearly identifiable presence of the fibrillar collagen type I in these tissues suggests that luteal collagen is different from that of the follicle, where the basement membrane collagen type IV predominates (see Introduction). The luteinization of the ovulated follicle must therefore involve either a change in collagen gene expression, or at least a substantial increase in the synthesis of fibrillar collagen. Such an alteration in the predominant collagen type, from membrane to fibril, is consistent with the loss of a membrane separating granulosa and theca cells and with the formation of the dense, mixed tissue that characterizes the corpus luteum. The cells responsible for collagen production in either ovarian structure remain to be identified, as does the proximal cause of the change in synthesis.

In summary, we have identified the fibrillar collagen type I as a component of the bovine corpus luteum and inferred from hydroxyproline measurements that collagen may contribute up to about one sixth of the luteal dry matter. The synthesis of collagen takes place during luteal development and maturation, supported by the concurrent accumulation of its biosynthetic cofactor, ascorbic acid. Luteolysis is associated with a preferential loss of non-collagenic material.

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