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Regional Differences in Water Content, Collagen Content, and Collagen Degradation in the Cervix of Nonpregnant Cows

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ABSTRACT

The cow could be a suitable model for studies concerning functional changes of the cervix. However, as in many species, the bovine cervix becomes softer in texture during the follicular phase of the estrous cycle compared to the luteal phase. In the present study, we explored if changes in the collagen network take place that could be responsible for this phenomenon and if regional differences in water content, collagen content, and collagen degradation along the cross-sectional and longitudinal axes of the cervix were present. Two groups of nonpregnant animals with different progesterone status were studied. One group (n = 11) was under high progesterone influence, and the other group (n = 12) was under low progesterone influence. The water content was derived from the weight of the samples before and after lyophilization. The collagen content and the ratio of collagenous to noncollagenous proteins (hydroxyproline:proline ratio) were determined by performing amino acid analysis on hydrolyzed samples using high-performance liquid chromatography. Collagen denaturation was quantified with a colorimetric assay by determining the amount of hydroxyproline released from samples treated with α-chymotrypsine. The water content of the superficial layer of the submucosa was always significantly ($P < 0.01$) higher than the water content of the deep layer in the vaginal, mid, and uterine segments, but this was unrelated to the progesterone status of the animals. No effect of the tissue layers or of the progesterone status of the animals on the collagen content was observed, but an effect of segment was noted. The collagen content (μg/mg dry wt) in the vaginal segment of the cervix was significantly higher than in the mid ($P < 0.05$) and the uterine ($P < 0.01$) segments. The hydroxyproline:proline ratio showed the same pattern as the collagen content. The percentage of collagen denaturation in the superficial layer was always significantly ($P < 0.01$) higher than that in the deep layer, but no effect of the progesterone status or of the segment along the longitudinal axis was seen. It is concluded that regional differences in collagen biochemistry are present in the cervix of nonpregnant cows, which may account for the difference in firmness of different parts along the circular or the longitudinal axis of the cervix. However, differences in texture of the cervix between the two groups of cows could not be explained by differences in the collagen content, percentage of collagen denaturation, or water content.

cervix, corpus luteum, female reproductive tract

INTRODUCTION

During the follicular phase of the estrous cycle, the cervix of the cow becomes markedly softer than during the luteal phase [1]. This is also the case in species such as the horse [1] and the dog [2]. By contrast, in the pig, the cervix is stiff during estrus and becomes softer during the luteal phase [3]. Also, at parturition, the cervix has to soften before it can dilate. This process is caused by biochemical events that lead to structural changes in the connective tissue of the cervix, especially in its collagen network [4]. Degradation of the collagen network in connective tissues involves cleavage of the collagen molecules by, for example, collagenases. This prerequisite cleavage enables the triple-helical structure to unwind (a process called denaturation). The cleaved molecules, however, remain incorporated in the collagen network because of its cross-links [5], which may lead to a network that is less resistant to mechanical forces. In the case of the cervix, such an impaired collagen network may lead to increased distensibility during parturition. Although general agreement exists regarding the importance of connective tissue changes during softening of the pregnant cervix, it is difficult to quantify these reported changes, such as collagen loss [6] versus increased collagen synthesis combined with increased synthesis of proteoglycans and hyaluronic acid [7, 8]. For example, an increase in collagen synthesis could lead to an increase in weight of the cervix, either because of growth of the complete cervix or because of an increase in collagen per unit weight of the cervix. During the estrous phase in women, an increased infiltration of mast cells and macrophages into the stroma occurs [9], which is similar to the infiltration of polymorphonuclear cells during cervical dilatation at parturition [6]. This makes it plausible that the same mechanisms that cause softening in the pregnant cervix close to parturition are also involved in the textural changes that take place during the estrous cycle.

The mammalian cervix is not a homogenous structure. Along the cross-sectional axis, at least five different layers are morphologically distinguishable. Variation in distribution of different layers and tissue components along the cross-sectional and longitudinal axes of the pregnant as

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well as the nonpregnant cervix have also been described in different species [3, 10–16]. Because of the morphological differences between the layers, functional differences in speed and extent of softening in different parts of the cervix might be expected. This fact has been clearly demonstrated in pregnant sheep by the observation that only the dense collagenous connective tissue changes into a soft gel when the cervix ripens [17]. In addition, it was demonstrated that the uterine end of the ovine cervix softened more than the vaginal end, even when the cervix was surgically separated from the uterus, indicating that the uterus itself has no influence on this process [18]. Similarly, in pregnant pigs, biochemical and biomechanical studies revealed that softening does not proceed to the same degree and with the same speed at different sites along the longitudinal axis of the cervix [19–21].

Few and contradictory data concerning the distribution of and changes in the collagen network are available for the nonpregnant cervix [22–26]. Petersen et al. [24] did not find any significant differences in hydroxyproline concentration between the vaginal and uterine ends of the human cervix. Neither did they find any differences in passive biomechanical properties between the vaginal and uterine ends or between preparations that were cut parallel or perpendicular to the cervical canal. This is in contrast to the report of Conrad and Hoover [22] involving rabbits; those authors observed significant differences in passive biomechanical properties between the vaginal and uterine ends. In addition, Conrad et al. [23] found a significant decrease in stretch modulus from the lumen to the outer wall of the human cervix.

Because collagen is a major component of the connective tissue, the distribution, content, and degree of degradation of the collagen may influence the texture of the cervix. In studies regarding functional changes in the connective tissue of the cervix, nonpregnant individuals many times are used as controls (against pregnant individuals), often without any regard for the stage of the estrous cycle. Softening of the cervix during the estrous phase has been reported in many species; therefore, it was our aim to see if this softening should be taken into account when using nonpregnant individuals as controls.

In the case of in vivo studies, sampling usually is possible only from the caudal cervix, although in some cases (mouse and rats), the whole organ is used. In other cases, specimens are collected during cesarean sections in women, in which case the localization of the biopsy may not be consistent. In those cases, it is important to know if the tissue obtained is representative of the rest of the cervix. In addition, in most species, ethical and anatomical restrictions limit us to taking one biopsy specimen per individual. Using the cow as an animal model for studies of the cervix might overcome some of these practical problems. In the present study, we aimed to assess if significant differences exist in the collagen network between animals under high progesterone (HP_4) or low progesterone (LP_4) influence, which may possibly explain the softening of the bovine cervix during the LP_4 phase of the estrous cycle, and if regional differences in collagen biochemistry are present along the cross-sectional or longitudinal axis of the bovine cervix.

MATERIALS AND METHODS

Reagents

Guanidium chloride (GuHCl), EDTA, iodoacetamide, α-chymotrypsine (αCT), 9-fluorenylmethyl chloroformate (FMOC), pyridoxine monohydrochloride, homoarginine, and the amino acid standard for collagen hydrolysates were obtained from Sigma (St. Louis, MO). Hydrochloric acid (37%, or 12M), sodium acetate trihydrate, sodium hydroxide (analytical grade, p.a.), acidic acid, 2-propanol, chloramine-T, dimethylaminobenzoaldehyde (DMBA), perchloric acid (60%), and hydroxyproline were purchased from Merck (Darmstadt, Germany). Heptafluorobutyric acid (HFBA) and citric acid were obtained from Fluka (Buchs, Switzerland). Acetonitrile (ACN) and pentane were obtained from Rathburn (Walkerburn, Scotland).

The concentrations of pyridoxine and homoarginine in the internal standard solution for amino acid analysis were 10 μM and 2.4 mM, respectively. The amino acid standard was diluted together with homoarginine in 0.1 M borate buffer (pH 8.0) so that the injected volume during high-performance liquid chromatography (HPLC) contained 250 pmol of proline and hydroxyproline, 25 pmol of cystine, and 100 pmol of homoarginine. Incubation buffer consisted of 1 mM iodoacetamide and 1 mM EDTA in PBS (pH 7.5). A solvent of 4 M GuHCl in incubation buffer was used for extraction of proteoglycans and soluble collagen. Digestion buffer was made by dissolving 1 mg/ml of αCT in incubation buffer. Stock buffer (pH 6.1) contained 50.44 g/L of citric acid, 117.76 g/L of sodium acetate trihydrate, and 34 g/L of sodium hydroxide p.a. Assay buffer was made by mixing stock buffer with 2-propanol and deionized water in a ratio 10:3:2 (v/v/v). Chloramine-T reagents contained 0.141 g of chloramine-T dissolved in 1 ml of 2-propanol, 1 ml of deionized water, and 8 ml of stock buffer. The DMBA reagents contained 4 g of DMBA in 2.5 ml of 2-propanol and 5.5 ml of 60% perchloric acid. The 200 μM hydroxyproline standard contained 26.23 μg/ml of hydroxyproline.

Tissue Collection

Intact uteri and ovaries were collected at a commercial slaughterhouse. Shortly after stunning, a jugular blood sample was collected from each cow. The uteri were dissected and placed in a bag filled with ice-cold isotonic saline (0.9% NaCl). Bags were numbered and put in an ice-filled bucket for transportation.

Based on the presence or absence of a corpus luteum (CL) or follicle and on their respective sizes, the specimens were divided in a nonluteal group and a luteal group. Selection of specimens for the nonluteal group was based on the following parameters: at least one ovary with a small and hard CL or no CL; a preovulatory follicle or ovulation stigma; a soft, swollen cervix; and abundant, clear mucus in the vagina. Selection of specimens for the luteal group was based on the following parameters: at least one ovary with a large and soft CL, with either follicle(s) present; a hard, firm cervix; and no mucus present in the vagina. In addition, possible cases of cystic ovarian follicles, noncycling animals, cases of endometritis, or recent calvings were excluded. Afterward, a finer selection was made into an HP_4 group and an LP_4 group based on serum progesterone levels, and these groups were further used in the present study.

Circular slices (thickness, ~5 mm) were cut from the vaginal, mid, and uterine part of the cervix (Fig. 1) and divided into smaller, wedge-shaped pieces for different types of analyses (see below). The uterine segment of the cervix was obtained just caudal to the site where the cervical mucosa changed into the uterine mucosa. The vaginal segment was obtained approximately 1 cm cranial from the external os of the cervix, and the midsegment was obtained halfway between the two former sites. The tissues from nonluteal animals were always softer and easier to cut through than the tissues from luteal animals. Samples were taken from the superficial stromal layer of the wedges that had been cut out of each circular slice, consisting of epithelium and the fibromatous tissue directly underneath it, and from the deep stromal layer, consisting of the more peripheral fibrous tissue (Fig. 1B). Samples were frozen in liquid nitrogen and kept at −80°C, until further processing. In summary, only luteal cervical samples from animals with a serum progesterone level of greater than 2 ng/ml were finally accepted into the HP_4 group. Only nonluteal samples from cows with a serum progesterone level of 0.5 ng/ml of less were finally accepted into the LP_4 group. The progesterone concentrations were measured by a validated, direct, solid-phase, ^{125}I RIA method [27]. Sensitivity of the assay was 47 pg/ml. The interassay coefficient of variation was 11% (n = 16), and the intraassay coefficient of variation was 7.5% (n = 20).

Isolation of Collagen from Cervical Tissue and Morphology

Tissues from the cervical mucosa of three animals (two luteal and one nonluteal) were incubated overnight at 4°C with 1 mg/ml of pepsin in 0.5 M acetic acid (tissue:enzyme ratio, 1:25 [wet wt/v]). The released collagen

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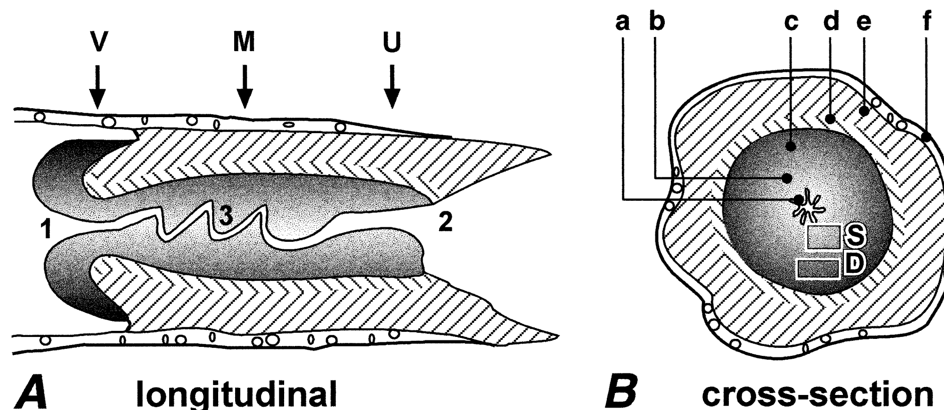

FIG. 1. Schematic pictures of the bovine cervix. **A**) Longitudinal section of the cervix. V, Vaginal segment; M, midsegment; U, uterine segment; 1, portio vaginalis (external os); 2 = uterine lumen; 3, cervical canal. **B**) Cross-section of the cervix. Small samples from the superficial (S) and deep (D) layers at each segment were used for biochemical analysis. a, Epithelium; b, superficial loose stromal band; c, deep dense collagenous layer; d, circular-oriented muscle layer; e, longitudinal-oriented muscle layer; f, serosal lining with a thin layer of very loose connective tissue and blood vessels underneath.

was subsequently loaded on 7.5% SDS-polyacrylamide gels, subjected to reduced interrupted gel electrophoresis, and stained with Coomassie brilliant blue as described previously [28]. Collagen types I and III, isolated from human placenta by pepsinization, were used as controls.

To study the general morphology, additional wedges of tissue, consisting of both mucosal and muscle layer from the vaginal, mid, and uterine segments of the cervix of four HP_4 and two LP_4 animals, were placed in 4% buffered formaldehyde for 48 h and embedded in paraffin. Sections (thickness, 6 μm) were mounted on glass slides and stained with periodic acid-Schiff and hematoxylin.

Tissue Preparation for Biochemical Analysis

From each cervix, six different sites were analyzed: vaginal superficial, vaginal deep, midsuperficial, middeep, uterine superficial, and uterine deep. For all 23 different animals (HP_4, n = 11; LP_4, n = 12), the six sites were analyzed, except for a few cases, as indicated in *Materials and Methods* and specified under *Statistical Analysis,* because the samples appeared to be too small for proper analysis. Each superficial and deep tissue specimen was divided into four smaller samples. Two of these samples were used to determine the percentage denaturation of the collagen in duplicate, and the other two were used for amino acid analysis in duplicate (see below).

Water Content

All tissue samples that were used for determination of the percentage denaturation and for the amino acid analysis were thawed for 2 h in isotonic saline, blotted dry, and weighed to obtain the wet weight. They were then lyophilized and weighed again to obtain the dry weight. After this, the water content could be calculated (wet wt − dry wt) and was expressed as a percentage of the wet weight. Consequently, the water content of each site for each individual animal was the mean of four samples. For all the sites, the number of animals tested in each group is specified in *Statistical Analysis.* The wet weight ranged from 50 to 200 mg, and the dry weight ranged from 6 to 37 mg. After lyophilization, the samples were further processed.

Analysis of Collagen Content

Collagen content was determined by measuring the amount of hydroxyproline in the duplicate samples according to the method of Bank et al. [29, 30]. The means of the duplicate samples were used for statistical analysis. Briefly, lyophilized samples were hydrolyzed in 6 M HCl for 24 h at 110°C and then dried. The samples were dissolved in an internal standard solution containing 2.4 μmol of homoarginine per 1 ml of water (to keep track of losses during the different steps of the procedure) and were diluted 5-fold with 0.5% (v/v) HFBA in 10% (v/v) ACN. The samples were then further diluted 50-fold with 0.1 M borate buffer (pH 8.0). Next, 200-μl samples were derivatized with 200 μl of 6 mM FMOC and extracted twice with 600 μl of pentane. Following this, 400 μl of 25% (v/v) ACN in 0.1 M borate buffer (pH 8.0) were added, and amino acid analysis was performed by HPLC. The content of the amino acids hydroxyproline and proline was derived from the chromatograms obtained. Collagen content was calculated based on hydroxyproline; it was assumed that collagen contains 300 hydroxyproline residues per triple helix and that the molecule has a molecular weight of 300 kDa.

The ratio of hydroxyproline to proline was calculated. The hydroxyproline:proline ratio of pure collagen type I is 0.858. Such ratios are not found in tissue hydrolysates but are always lower, because noncollagenous proteins contribute to the released proline pool. As such, the hydroxyproline:proline ratio reflects the ratio of collagenous to noncollagenous protein.

Because some of the hydrolysates were lost during drying because of technical problems, it was not possible to analyze all six sites for each animal for the collagen content and hydroxyproline:proline ratio. The number of animals tested for each site is specified in Table 1 for the two progesterone groups.

Analysis of Collagen Degradation

The samples were processed in duplicate as described previously [5]. Briefly, proteoglycans and soluble collagen were removed from the tissue by extracting tissue samples twice for 24 h with 4 M GuHCl in incubation buffer at 4°C. After three washes in incubation buffer to remove GuHCl, the denatured collagen in the insoluble tissue matrix was digested overnight at 37°C in 1 ml of digestion buffer. The supernatant containing the αCT-solubilized collagen fragments was removed quantitatively and diluted 1:1 with 12 M HCl. The remaining tissue was immersed in 800 μl of 6 M HCl. Supernatant and residual tissue were hydrolyzed at 110°C for 24 h and dried. After drying, the hydrolysates were dissolved in 1 ml of water. The hydroxyproline concentrations of the hydrolysates from the supernatant and the remaining tissue were measured by a colorimetric method according to the principles of Stegeman and Stadler [31] and as described by Creemers et al. [32]. In short, the hydrolysates of the supernatant and of the remaining tissue were diluted 10- and 50-fold, respectively. From these samples, 60 μl were pipetted into a well of a polystyrene microtiter plate, after which 20 μl of assay buffer and 40 μl of

TABLE 1. Number of animals used for each site of the cervix, specified for each group and for the different types of biochemical analysis performed on the nonpregnant bovine cervix.

	Group	Vaginal superficial	Vaginal deep	Midsuperficial	Middeep	Uterine superficial	Uterine deep
Water content	HP_4	11	11	11	11	10	10
	LP_4	12	12	12	12	12	12
Collagen content (hydroxyproline:proline)	HP_4	7	7	7	7	5	5
	LP_4	12	12	10	10	10	10
% Collagen denaturation	HP_4	11	11	11	11	9	10
	LP_4	12	12	12	12	11	11

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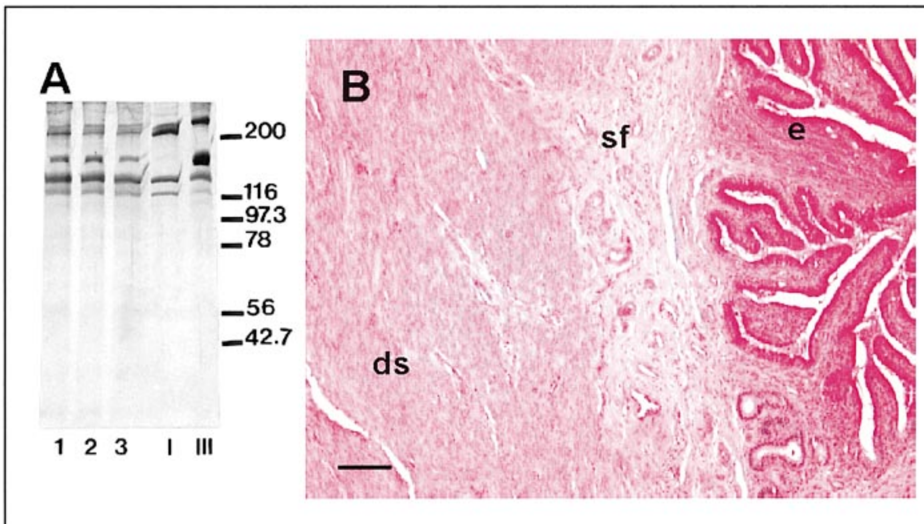

FIG. 2. **A**) Collagen type in the cervix of three cows was analyzed by SDS-PAGE. The numbers 1, 2 and 3 are the samples from three different cows. The numbers I and III are the controls for collagen type I and type III, respectively. **B**) Photomicrograph from the mucosal area of the cervix. Periodic acid-Schiff/hematoxylin staining shows the deep stromal layer (ds), superficial loose stromal band (sf), and columnar epithelium (e). Bar = 14 μm.

chloramine-T reagent were added. After a 20-min incubation at room temperature, 80 μl of DMBA reagent were added to the samples and then carefully mixed. Subsequently, the plate was closed with a lid and placed in a small water bath in an incubator at 60°C for 25 min. After this, the plate was cooled by placing it for 5 min in a water bath containing cold water, which was refreshed once during that time, and the extinction was measured at 570 nm on a Titertek multiscan MCC/340 (Titertek, Huntsville, AL). A hydroxyproline standard series (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 μM) and blanks (water) were included in the measurements. The extinction of the standards and the samples was first corrected for the extinction of the blanks. If the extinction of the samples was higher than that of the maximum dilution of the standard series, they were diluted further and then measured again.

The percentage denaturation of the collagen in the samples was calculated as

$$\% \text{ Denatured collagen} = (A/A + B) \times 100\%$$

where A is the extinction of the supernatant hydrolysate multiplied by the dilution factor and B is the extinction of the tissue hydrolysate multiplied by the dilution factor. The means of the duplicate samples were used for statistical analysis. The number of animals tested for each site is specified in Table 1.

Statistical Analysis

The number of animals used in each group for each site of the cervix is specified for the different biochemical analyses in Table 1. All data were analyzed with the general linear model procedure of the SAS software (SAS Institute, Inc., Cary, NC) using the following model:

$$Y_{ijk} = \mu + P_{4_i} + \text{Animal}_j(P_{4_l}) + \text{Segm}_j + \text{Depth}_k + (\text{Segm}_j \times \text{Animal}_j(P_{4_l})) + (\text{Depth}_k \times \text{Animal}_j(P_{4_l})) + e_{ijk}$$

where Y_{ijk} is the dependent variable; μ is the overall mean; P_{4_i} is the progesterone level (i = high or low); Animal(P_{4_l}) is the animal effect (j = 1–23), nested within the progesterone level; Segm_j is the segment of the cervix (j = vaginal, mid, or uterine); Depth_k is the cross-sectional depth (k = superficial or deep); and e_{ijk} is the residual error.

The differences between the progesterone groups (HP_4 vs. LP_4) were tested against the variation between animals. The variation between segments and the variation because of depth of sampling were tested against the $\text{Segm}_j \times \text{Animal}(P_{4_l})$ interaction and the $\text{Depth}_k \times \text{Animal}(P_{4_l})$ interaction, respectively. All other interactions were not significant. All data are presented as the mean ± SEM. Significance was accepted at $P < 0.05$.

RESULTS

Typing of Collagen: Microscopic and Gross Morphology

The results from the electrophoresis showed that both collagen type I and collagen type III are present in the bovine cervix (Fig. 2A).

The cervices of the LP_4 group felt softer by palpation and appeared to be more hydrated than those of the HP_4 group. Macroscopically, it was observed that at the vaginal side, the muscle layer was relatively thinner compared to the uterine side, whereas the connective tissue part was relatively thicker than at the uterine side. Although quantitative analysis was not performed, microscopy appeared to confirm this observation.

Between the lumen and the muscle layer of all cervices, a softer part and a more firm part underneath could be discerned by palpation. Microscopically, a subepithelial, superficial, loose stromal band and a deep, dense collagenous layer of the submucosa could be clearly distinguished in all cases (Fig. 2B).

Water Content

A significant relationship was observed between the depth of the tissue layer and the water content ($P < 0.0001$). The water content of the superficial layer was significantly higher than the water content of the deep layer in all segments ($P < 0.01$) (Fig. 3). However, no effect of the progesterone status of the animals or of the segment along the longitudinal axis of the cervix on the water content was seen.

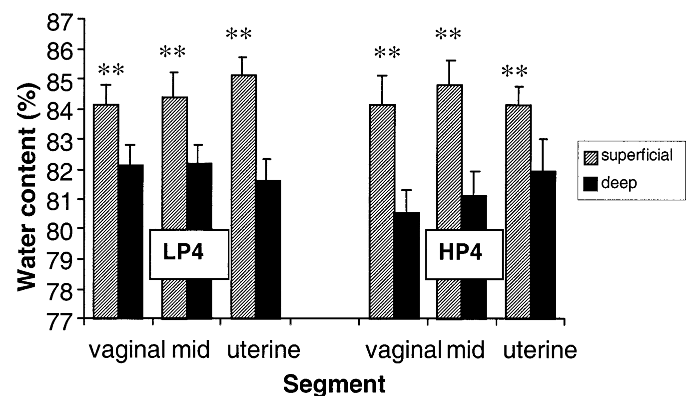

FIG. 3. Water content (% wet wt, mean ± SEM) in three different segments along the longitudinal axis of the bovine cervix. Asterisks indicate a significant difference in water content between the superficial and deep stromal layers of each segment (**$P < 0.01$).

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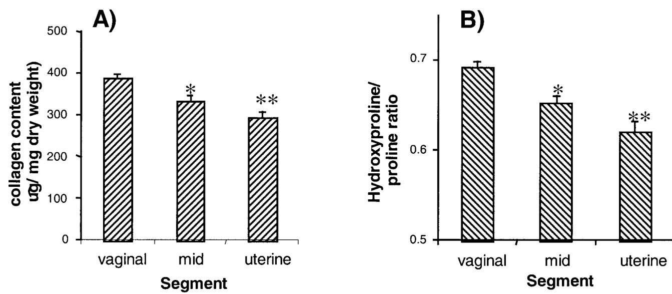

FIG. 4. Collagen content (**A**; mean ± SEM) and ratio of collagenous to noncollagenous protein (**B**; hydroxyproline:proline ratio, mean ± SEM) of pooled values of the superficial and deep layers from both the HP_4 and LP_4 groups in three different segments of the bovine cervix. Significant differences compared to the vaginal segment are indicated by asterisks (*$P < 0.05$, **$P < 0.01$).

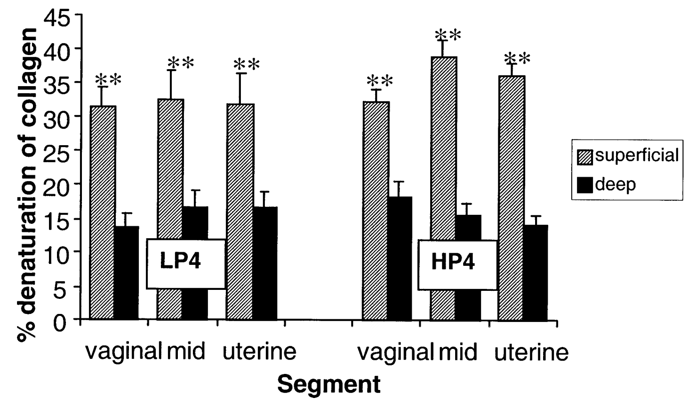

FIG. 5. Percentage of denaturation of the collagen (mean ± SEM) in three different segments along the longitudinal axis of the bovine cervix. Within the same segment, asterisks indicate a significant difference in the percentage denaturation between the superficial and deep stromal layers (**$P < 0.01$).

Collagen Content and Ratio of Collagenous to Noncollagenous Proteins (Hydroxyproline:Proline)

A significant effect of segment ($P < 0.0001$), but not of depth of the tissue layer or of progesterone status, on the collagen content was observed. Because no differences were seen between the HP_4 and LP_4 groups or between the tissue layers, the data were pooled to analyze the effect of segment. The vaginal segment had a significantly higher collagen content than the mid ($P < 0.05$) and uterine ($P < 0.01$) segments (Fig. 4A).

Similarly, no relation was found between the tissue layers and the progesterone status on the hydroxyproline:proline ratio, but an effect of the segment ($P < 0.0001$) was observed. Therefore, the data were also pooled to analyze the effect of segment. At the vaginal segment, a significantly higher hydroxyproline:proline ratio was found compared to that at the mid ($P < 0.05$) and the uterine ($P < 0.01$) segments (Fig. 4B).

Percentage Denaturation

A significant relationship was observed between the depth of the tissue layer and the percentage denaturation ($P < 0.0001$), but no effect of progesterone status or segment was found. In both groups, the percentage denaturation was significantly higher in the superficial layer than in the deep layer of all segments ($P < 0.01$) (Fig. 5).

Also, a positive linear relationship was found between the water content and the percentage denaturation ($R = 0.36$, $P = 0.0001$). The correlation was 0.46 ($P = 0.0001$) (Fig. 6A) for the HP_4 group and 0.31 for the LP_4 group ($P = 0.0099$) (Fig. 6B); this difference was not significant (heterogeneity of regression).

DISCUSSION

The present study demonstrates, to our knowledge for the first time, that significant differences in water content and collagen degradation are present between the superficial and the deep stromal layers of the submucosa of the cervix of nonpregnant cows in both the presence and the absence of a functional CL. Moreover, differences in collagen content and the ratio of collagenous to noncollagenous proteins were found between the vaginal and uterine segments of the cervix. Our finding that the bovine cervix contains both collagen type I and type III is in agreement with the observation in the human cervix [33].

A decreasing collagen content was found from the vaginal toward the uterine side of the cervix in both the LP_4 and the HP_4 groups, whereas along the cross-sectional axis, no differences were observed in collagen content between the superficial and deep layers. The subjectively observed difference in softness between the two layers during collection of the cervix samples cannot be explained by differences in collagen content. Our results, therefore, are in line with the reported absence of a relationship between the phase of the cycle and collagen content, as observed in hysterectomy specimens of the human cervix [24], but not with the reported fluctuations in total collagen content of the cervix during the estrous cycle of the mouse [34].

Major differences in the collagen content of the cervix exist between species. When the collagen content as found in the present study is expressed as a percentage of dry weight, it measures approximately 38% at the vaginal side and approximately 29% at the uterine side. These figures are higher than those reported for the mouse (16%) [34], are within the same range as those reported for the sheep (44%) [35] and the rabbit (42%) [13], but are much lower than those reported for the rat (60–70%) [36] and the human (50–85%) [7, 37, 38]. In contrast to our results, the human cervix appears to have a higher collagen content at the uterine end than at the vaginal end [7], although yet another report claimed that no differences are found in collagen content [24] along the longitudinal axis of the nonpregnant human cervix. However, expressing the collagen content relative to dry weight, as Leppert and Yu [7] did, may give different results, as when expressed relative to wet weight, as Petersen et al. [24] reported.

Our findings indicate that the soft appearance of the superficial stromal layer may be explained by a higher percentage of denatured collagen. This suggests that the degree of denaturation of the collagen network could be of more importance than the collagen content itself for determining the texture of the stromal tissue of the cervix. In addition, an increased digestion of collagen may have led to an increase of the amount of smaller soluble collagen fragments. These may have been washed out during the GuHCl extraction (in the denaturation assay), and thus, they may also be responsible for the difference in rigidity between the cervices of the two groups of animals. This difference would not become apparent by only measuring the total collagen content, as we did. Structural changes do not correlate well with changes in collagen content or concentration, as was demonstrated by Junqueira et al. [39], who observed, by means of histochemical analysis, that the loss

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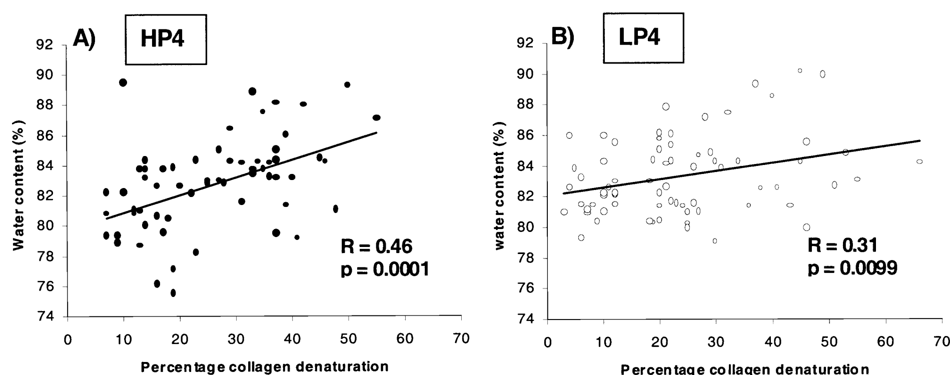

FIG. 6. Correlation between the percentage of denatured collagen and the water content of the cervix in the HP_4 group (**A**) and the LP_4 group (**B**).

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of structure in the collagen network (fiber length and orientation) in parturient women was much more pronounced than the amount of collagen would suggest. The observations of Conrad et al. [23] that different zones along the circular axis of the nonpregnant human cervix had the same collagen content whereas the stretch modulus decreased significantly from the lumen toward the outer wall also support our findings. Reported differences in collagen extractability after pepsin digestion in the softened human cervix [40–44] also suggest that degradation of the collagen network alters its structure so that it can be more easily extracted.

A significant increase in the water content of the porcine cervix occurs shortly after a 2-day exposure to estrogens, and similar results were found in pigs that had been given estrogen daily over a more prolonged period [45]. These observations suggest that an increase in circulating estrogens stimulates the attraction of water into the cervical tissue. Other authors [46], however, found no effect of estrogen on the water content of the porcine cervix, when estrogen without progesterone was administered every other day during a 2-wk period. On the basis of ovarian morphology, it can be assumed that the cows in our LP_4 group were slaughtered either during or shortly after a period of estrogen dominance. In a similar group of cows, we have found that in animals under LP_4 influence, tissue concentrations of estrogens are significantly higher than in cows under progesterone dominance [47]; thus, the stimulatory effect of the estrogens on the cervical water content might still be present. Nevertheless, our present results did not show significant differences in water content between the LP_4 and HP_4 groups. On the other hand, the observations that the superficial layer clearly felt softer by palpation than the deep layer and that it also had a significantly higher water content indicate that the water content has an important influence on the texture of the cervix. More studies are therefore needed to elucidate the role of estrogens in determining the water content of the stromal tissues of the bovine cervix.

Apparently, the observed differences in water content between the superficial and the deep stromal layers of the cervix in both the HP_4 and LP_4 groups are associated with the differences in collagen denaturation, although we did not find an equally high correlation as that reported by Bank et al. [29, 48], who found that the swelling properties of cartilage are highly correlated with the amount of denatured (degraded) collagen. However, one should realize that the concentration or composition of other connective tissue constituents other than collagen that are present within the cervix can influence the texture. The differences between the hydroxyproline:proline ratio of the cervical segments showed the same pattern as the collagen content (Fig. 4). In human cartilage, the hydroxyproline:proline ratio is negatively correlated with the total glycosaminoglycan concentration in the tissue (unpublished results). If the same applies to the cervix, this would indicate that the decreasing hydroxyproline:proline ratio from the vaginal to the uterine segments is indicative of a similarly oriented increase in glycosaminoglycan content, which might cause differences in the texture of the cervical tissue [6, 49]. As judged by their similar collagen content and hydroxyproline:proline ratios, the total glycosaminoglycan content of the deep stromal layer is probably not different from that of the superficial stromal layer. However, it can be hypothesized that the composition of the glycosaminoglycan content in the superficial layers is more in favor of glycosaminoglycans with a higher water-binding capacity as compared to the deep layers. For example, hyaluronic acid may contribute more to the total dry weight of the connective tissue in the superficial stromal layer of the cervix and may also be responsible for attracting more water to that layer [49]. In line with this hypothesis, the ratio of glycosaminoglycans with higher water-binding capacity to those with lower water-binding capacity could be higher in animals around estrus. Similarly, decorin, which stabilizes the collagen network [50], may be present at lower concentrations in estrous animals or may play a role in the difference of texture between the superficial layer and the deep stromal layer. This remains to be investigated for the cow.

In the present study, it was found that significant differences exist in the percentage of collagen denaturation as well as in the water content along the cross-sectional axis of the bovine cervix. The fact that the percentage of collagen denaturation of the superficial layer is higher than that in the deep layer, in spite of both layers having an equally high collagen content, suggests a role for the percentage of collagen denaturation in defining the texture of the cervical tissue. The results also showed significant differences in collagen content along the longitudinal axis of the cervix. These findings are relevant for the interpretation of results obtained from in vivo sampling of the cervix, when only the caudal (vaginal) part of the cervix can be reached and when different studies with different sampling regions are compared. However, differences in texture of the cervix between the two groups of cows could not be explained by differences in the collagen content, percentage of collagen denaturation, or water content.

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