# Collagen Changes in Rat Cervix in Pregnancy—Polarized Light Microscopic and Electron Microscopic Studies (43908)

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*Abstract.* **The structural arrangement of collagen fibers in cervical ripening was studied in normal pregnant rats by picrosirius red staining and polarized light microscopy. The macromolecular arrangement of collagen fibers in the cervices of nonpregnant controls and in firm and rigid cervices of rats in early pregnancy (1–10 days of gestation) were optically anisotropic and had birefringence and a positive sign of elongation when examined by polarized light microscopy. The findings indicated that the structure of these collagen fibers was assembled from well-packed parallel collagen molecules. The direction of fibrous formation was arranged with regularity. In contrast, most of the collagen fibers in the soft cervices were optically isotropic. The fibers were fragmented and had a structure with discontinuous birefringence. Disarray and disorientation of the collagen fibers was found in the soft cervices. These collagen fibers changed their direction of formation. The disorganization of these collagen fibers might have a major impact on weakening the tensile strength of the cervix. Thus, we conclude that the processes of rearrangement of collagen fibers might be an important process in the cervical ripening. Electron microscopic studies suggest that in the focal hydrolytic processes of collagen and other matrix components degradation by lysosomal and phagosomal vesicles were associated with atrophic smooth muscle cells and fibroblasts of the cervices. Hydrolases released from lysosomes from these apoptotic cells may presumably be one of the processes in the remodeling of collagen structure.** [P.S.E.B.M. 1995, Vol 209]

The cervical changes of pregnancy are the enlargement of its geometric structure, the softening of tissue, and the beginning of dilation of the lumina. This remodeling process allows the easy delivery of the fetus. In contrast, the cervices in nonpregnant animals have a rigid structure. In early pregnancy, the cervix is firmly closed and functions as a structural barrier to maintain the conceptus in the uterus.

The changes of cervical collagen in pregnancy have been extensively investigated (1–12), since collagen is a predominant fibrous element in the cervix and plays a major role in the mechanical properties of the tissue (13). However, the exact nature of the rearrangement of the cervical collagen is still incomplete.

Cervical softening, dilatation, and alteration of the tensile properties of the uterine cervix in gestation are often considered to be synonymous terms. This may not be true. Cervical softening is independent of cervical dilatation. In pregnant women, softening occurs prior to parturition and may occur in normal gestation as early as 36 weeks (14). Dilatation, on the other hand, most commonly occurs in association with intermittent contractions and is clinically distinct from cervical softening (15). Certainly, the clinically softening cervix still remains closed for several weeks prior to parturition. In general, stiff, rigid tissue has high tensile strength; tissue soft to digital touch can retain tensile strength, also. It is the orientation of the collagen fibers that causes the softness to palpation (16). Thus, cervical softening may not occur simultaneously with loss of tensile strength.

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Uterine cervical softening in pregnancy has been explained to some degree by changes in the biochemical components of this tissue (17–22). Elevation of water concentrations (23–26), changes of collagen concentration and increase of soluble collagens (1, 7, 8, 12), alterations in distribution of proteoglycans in the cervices (27–29), and changes of soluble proteins and enlargement of cervical mass (26) have all been reported. Although no single biochemical component can explain cervical ripening, a combination of these changes probably contributes to alteration of the mechanical properties. Of the biochemical components of the cervix, collagen has been the most extensively studied. Decrease of collagen (hydroxyproline) concentrations in ripe cervices has been demonstrated by various investigators (2–11, 26, 30). There is also an increase in extractable collagen (1, 7, 9, 10). An elevation of serum collagenase activity in pregnant women linked to the cervical ripening has been reported (29). All these findings suggest to some workers that changes of cervical collagen may be attributed to the activity of collagenases or related proteolytic enzymes (5, 7, 31–39). Collagen fibers are widely distributed throughout cervical tissue, accounting for 40%–60% of cervical total protein, and are the major insoluble fiber (26).

The enzymes degrading intact collagen fibers are present in polymorphonuclear leukocytes and macrophages (36, 37), which release collagenases and proteases and degrade the fibers directly or during phagocytosis. Infiltration of neutrophils in the cervical tissues of pregnant women has been reported by several investigators (32, 33, 37–41). In human subjects, pelvic infection is frequently associated with premature labor and cervical dilatation (42, 43). Softened or ripe tissues are morphologically similar to those with inflammation of the cervix, or cervicitis (1). Therefore, it has been postulated that an inflammatory reaction accounts for a large component of cervical ripening, due to action of the collagen degrading enzymes. (31, 38, 41).

However, we find that rat cervix in late gestation does not exhibit signs of inflammation or necrosis at any time in normal pregnancy, including late gestation. Furthermore, there is no infiltration of neutrophilic leukocytes or other white cells in the immediate vicinity of collagen fibers in these cervices throughout gestation (44).

In addition, studies of the secretion of collagenase in pregnant rat cervical tissue have failed to demonstrate a significant increase of the collagenase activity near term (30). Similar reports were published on studies in guinea pigs (40) and sheep (41). Therefore, we think there are some other explanations for the changes of collagen in the remodeling cervices. Histological and biochemical changes in rat cervix during pregnancy were described in 1968 by Datta *et al.* (45), when they observed the disorganization of connective tissue bundles along with marked alterations of cervical morphology at 21 days of pregnancy. They concluded that the changes were primarily under the influence of estrogen. More recently, Lee *et al.* (46) described the structural changes of collagen fibers and elastic fibers by morphometric analysis of the cervices of the pregnant rats. The rats were treated with antibody against relaxin, and they demonstrated relaxin is an essential hormone for histological changes apparently associated with cervical softening.

Because these studies describe the histological alterations of cervical tissue under conventional light microscopy, the underlying fine structural change of the described disorganization of the collagen fibers cannot be completely determined. These studies, therefore, cannot resolve the issue of whether or not the predominant change in cervical collagen is rearrangement and realignment of fibrils within the fibers, or if the major alteration is degradation of the collagen. Aspden (13) hypothesized, based on an x-ray diffraction analysis of the fiber orientation, that collagen fibers decreased in fiber length and decreased in fiber alignment or organization in pregnancy. Collagen fibers need to have a critical length of 20 μm to maintain tensile strength, and the x-ray diffraction analysis of the cervix demonstrates that the fiber length decreases below that critical length as pregnancy advances to term.

The purpose of this study was to determine if this hypothesis of structural transformation of the collagen fibers could be verified. We examined the optical anisotropy of collagen fibers in the cervices of pregnant rats with the polarized light microscope using a staining technique with anionic picrosirius red dye (47–50). This staining technique enables the determination of the birefringence of the tissue studied, thus demonstrating the orientation and alignment of fibrous units within the collagen fiber. Using this technique, we noted a pronounced disarray in the collagenous structure in the cervices of pregnant rats. By contrast, in nonpregnant or rigid cervices, the collagen fibers were well packed, and fibrous units appeared to be arranged in an orderly manner. We also studied the tissue by light microscopy using common histological stains.

In addition, we have examined the ultrastructural changes of collagen fibers by electron microscopic examination and determinedcollagen fiber disarray and a sporadic degradation of collagen fibers.

## Materials and Methods

Holtzman Sprague-Dawley rats (timed-pregnancy, first pregnancy, virus free) were purchased from Holtzman Lab Animals (Madison, WI). For pregnant rats, animals with gestations of 5, 10, 13, 15, 18, 19, 20, and 21 days were used ($n$ = 4 per group). Nonpregnant virgin females, 72 days of age and weighing approximately 250 g each, unselected for day of estrous cycle, were used as controls ($n$ = 3 per group).

The animals were sacrificed and the cervices were removed for study. For light microscopic studies the tissues were fixed in a 3% buffered formalin solution (sodium phosphate, 0.013 *M*, pH 7.5). The formalin fixed tissues were dehydrated in ethanol, cleared, and infiltrated in paraffin using an automatic tissue processor. All cervical tissues were embedded in paraffin blocks in preparation for cross-section and then cut approximately 0.3 mm from the external os of the cervix or cut across at the midcervix.

**Staining for Light Microscopy.** Paraffin sections (4 microns) were stained with hematoxylin and eosin, Gomori's trichrome, or van Gieson's stain for collagen fibers. These sections were comparisons for the picrosirius red stained tissues.

Picrosirius red F3BA staining technique was carried out according to the method of Junqueira *et al.* (32). Sirius red F3BA (C. I. No 35780; Pfaltz & Bauer, Waterbury, CN) was dissolved at 0.1% (w/v) in saturated aqueous picric acid solution, resulting in a solution with a pH of 1.4. The paraffin sections were cleared with xylene and hydrated with a series of graded ethanols and water. The sections were stained with the picrosirius red F3BA solution for 1 hr at room temperature. The sections were cleared with 0.01 *N* HCl, dehydrated with ethanol (three times), cleared in xylene, and mounted with Permont $\eta_D = 1.519$ (Fisher Scientific, Pittsburgh, PA).

**Light Microscopy.** A Nikon microscope (Nikon OPTIPHOT) equipped with a polarized analyzer and a first-order red compensator was used. For photography and for assessing birefringence, pleochroism, and signs of elongation, an Olympus Model BH-2 polarized light microscope equipped with a first order red compensator (a retardation of 500 nm) was used.

**Electron Microscopy.** In the electron microscopic study, the tissues were fixed with a 2% paraformaldehyde/0.2% glutaraldehyde in 0.01 *M* sodium phosphate-0.85% NaCl solution (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in polydbed-epon-812 (Polysciences, Warrington, PA). Thin sections were stained with lead citrate and uranyl acetate and examined with a Philip CM12 transmission electron microscope.

## Results

**Collagen Fibers in Normal Nonpregnant or Pregnant Cervices.** Under light microscopy, in the cervices of nonpregnant controls and in the cervices of rats in early pregnancy (less than 10 days of gestation), the fibrous collagen fibers found in the smooth muscle layers of the cervices were well organized and densely packed, as described previously (44). Collagen fibers were found in three distinct regions of the cervix. Those collagen fibers in the outside layer and the area adjacent to the canal were both oriented parallel to the length of the canal, and those collagen fibers in the middle region were arranged in multiple circular layers around the cylindrical cervix. Most of these collagen fibers were arrayed parallel to the smooth muscle cells. Among the three zones, those fibrous structures in the middle region changed most significantly during the course of cervical softening. Within the collagen fibers, those adjacent to the faciculi of smooth muscle cells became particularly disorganized (Fig. 1).

This structural arrangement was seen in the histological sections stained with Gomori's trichrome and in those stained with picrosirius red for collagen fibers (Figs. 1 and 2). These findings were also observed in the sections stained with hematoxylin and eosin (data not shown).

**Birefringence of Collagen Fibers Stained with Picrosirius Red.** In the firm, rigid cervices of early pregnancy, the collagen fibers were oriented with their

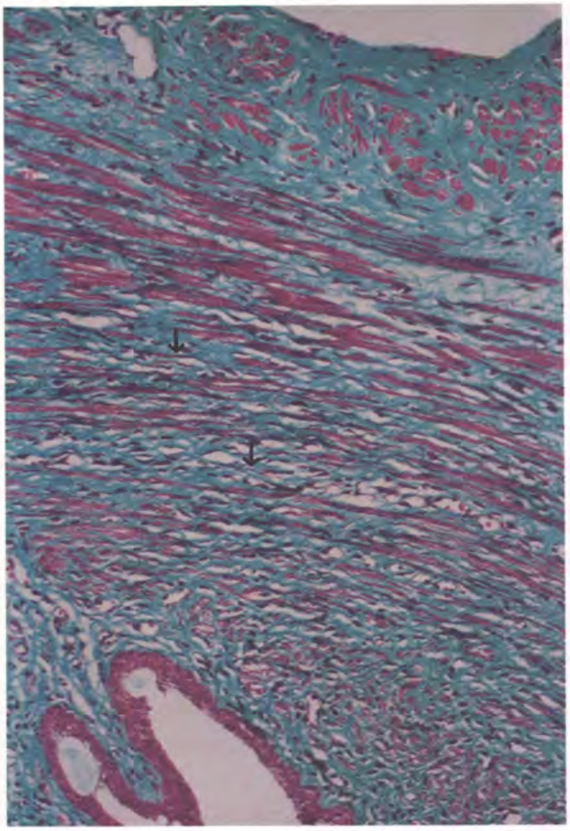

**Figure 1.** Cervical section of nonpregnant control rat stained with Gomori's trichrome stain for collagen (×175). The cross-section was obtained at the region 0.3 mm from the external os. The fasciculi of smooth muscle cells are seen in the myometrium (arrow), and the collagen fibers are in parallel arrangement between the fasciculi. The collagen is stained blue-green color and the smooth muscle cells are stained red.

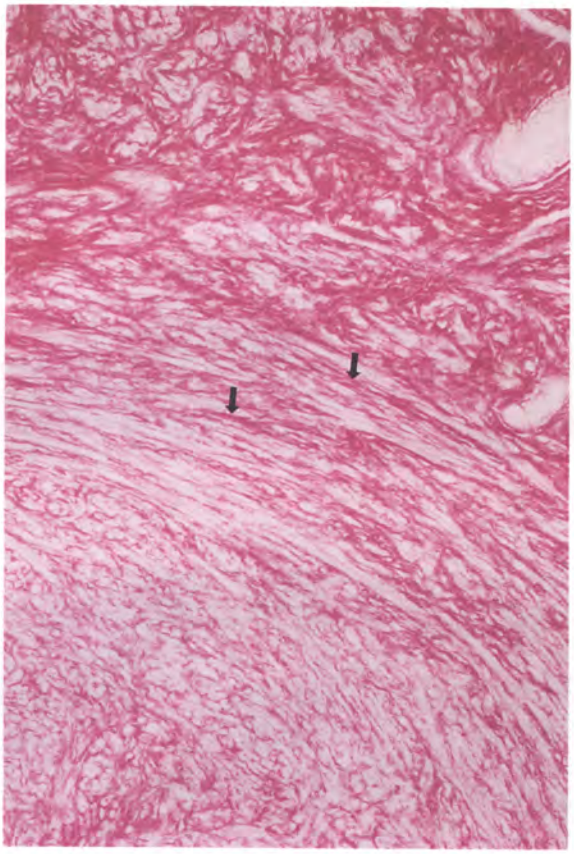

**Figure 2.** Cervical section of nonpregnant control rat stained with the solution of picrosirius red F3BA dye and photographed under normal light (×175). The cross-section was obtained at the junction adjacent to the vaginal wall and *ca.* 0.3 mm from the external os. Collagen fibers in parallel arrangement are seen in the myometrium (arrow) of the cervix.

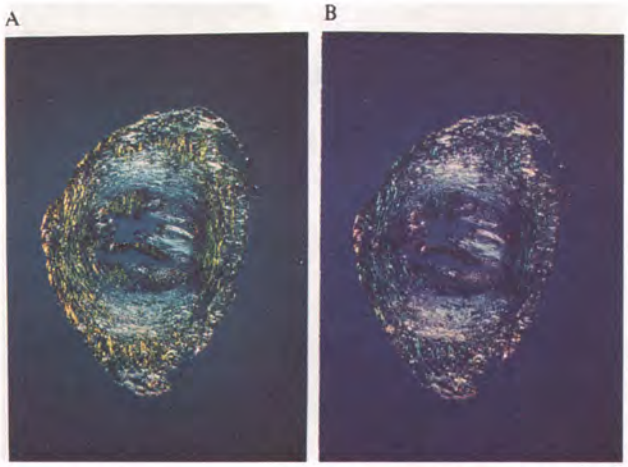


**Figure 3.** (A) Cervical section of nonpregnant control rat stained with the solution of picrosirius red F3BA dye and photographed under a polarized light microscope (×17). The polarized light was aligned from east to west. The well-organized collagen fibers show birefringence. The long axis of the collagen fibers located perpendicular to the light plane show bluish green color, but those fibers running parallel to the light plane show yellow color. (B) The same section (cervix of nonpregnant control rat) as that of Panel A except the long axis of the collagen fibers are rotated 90°. Both panels illustrate the birefringence of the collagen fibers. The long axis of the collagen fibers located perpendicular to the light plane and the fibers appear to be yellow, but those fibers running parallel to the light plane show bluish green.

long axes parallel to the direction of the circumference of the cylindrical cervix, and were seen under a normal light microscope on the sections stained with picrosirius red (Fig. 2). When these stained sections were examined under polarized light, the collagen of the rigid cervices were oriented as a bundle of long fibers and showed a homogeneous birefringence. These fibers showed a bluish green color when the long axes of the fibers were oriented perpendicular to the plane of polarized light (Fig. 3A). Normally, the polarized light microscope was aligned with its polarity to the east-west orientation. However, when the long axes of the fibers were oriented parallel to the plane of polarized light, these fibers showed a yellow color (Fig. 3B). This observation indicated that the refractive index of the collagen fibers at the parallel position of the fibers was higher than that at the perpendicular position, $\eta_{\parallel} > \eta_{\perp}$. Thus, these collagen fibers had a positive sign of elongation (48, 49).

These optical properties are characteristic of the anisotropic structure of oriented collagen fibers, and not of collagen molecules with an amorphous form or with loosely packed ill-oriented fibrils (50), which are optically isotropic. Thus, the collagen fibers of the rigid cervices were formed with well-packed collagen fibrils. The direction of formation of the collagen fibers in the rigid cervices showed a specific pattern of regularity, which normally was oriented parallel to the fasciculi of smooth muscle cells or fibroblasts (Fig. 4).

In the softening cervices (18–21 days of pregnancy), the structure of the collagen fibers adjacent to circular sphincteric smooth muscle cells changed to fragmented fibrous networks (Fig. 4 and 5). These collagen fibers were thinner than those of early pregnancy or of the controls. Under polarized light with the picrosirius red staining, most of these fibers were oriented with a varied and disarrayed birefringence.

Under polarized light the collagen fibers of the softening cervices showed the presence of birefringence, but with a discontinuous appearance (Fig. 6). The fibers, when oriented perpendicular to the polarized light plane, showed interrupted bluish green color. Those fragmented fibers, when oriented parallel to the polarized light plane, had a yellow color. These fragmented fibers occupied the intrafibrous spaces of the bluish green parallel fibers, and were oriented in a different direction (perpendicular to the array of the yellow collagen fibers).

Thus, these collagen fibers appeared to be dilated and dispersed randomly without having any direction

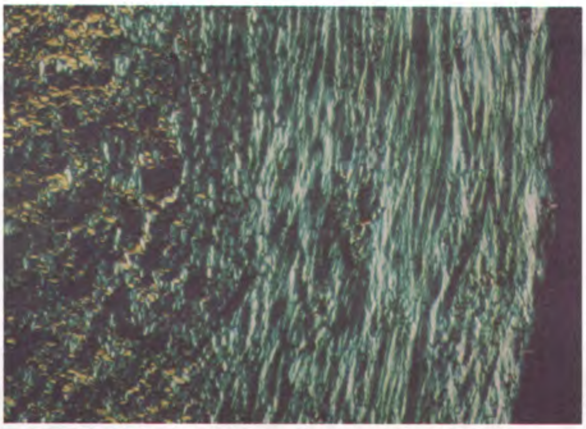

**Figure 4.** The same section and same staining (picrosirius red F3BA) as that of Figure 3 (cervix of nonpregnant control rat), and photographed under polarized light except higher magnification (×175). The collagen fibers are arranged with a specific regularity, and the fibers are formed parallel with the longitudinal oriented smooth muscle cells.

**Figure 5.** Cervical section of a pregnant rat at 20 days of gestation, stained with the picrosirius red, and photographed by a polarized light microscope (×17). The collagen fibers are fragmented and show discontinuous birefringence.

in the collagen bundle. This disarray results in a pattern of interrupted birefringence in each direction of the axis. In addition to these disoriented collagen fibers, there were numerous fragmented small fibrils which showed no birefringence. These fibrils probably were not formed into the fibrous structure (Fig. 6).

Incidentally, light microscopic examinations of the cervical tissues of pregnant rats from 0 through 21 days of gestation revealed no sign of inflammation, such as vasodilatation, exudation of plasma, and immigration of polymorphonuclear leukocytes into these tissues (data not shown).

**Electron Microscopic Findings.** The observations by light microscopic studies on the structural changes of collagen in soft cervices were supported by the electron microscopic study. It showed loosely packed (Fig. 7) collagen fibers separated from each other and the numerous noncollagen materials that were seen in the extracellular matrix spaces. Aggregates of microfibrils were also observed in the vicinity

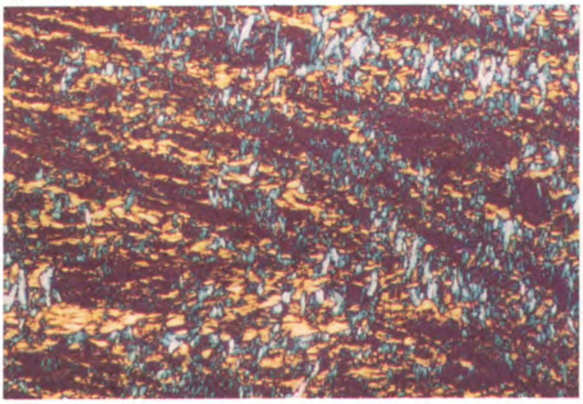

**Figure 6.** Cervical section of pregnant rat at 20 days of gestation, stained with picrosirius red, as in Figure 5 except with higher magnification (×175). The fragmented collagen fibers dispersed in all directions.


**Figure 7.** Electron micrograph of cervical tissues obtained from pregnant rat at 21 days of gestation (×23,000). Collagen structure adjacent to smooth muscle cells (S) appear to be loosely packed. The collagen fibers show disarray in their orientation, and they seemingly estrange each other. Phagosomes/lysosomes show clear spaces (arrows) in the fibrous structure of the extracellular space.

of the collagen fibers, which has been reported by other investigators (31).

The collagen fibers were disarrayed, and most of the fibrous long axes were poorly oriented and not parallel to the fasciculi of smooth muscle cells (Fig. 7). They were dissociated from the smooth muscle fasciculi in the cervix, while those collagen fibers of the unripe pregnant or nonpregnant cervices were closely packed with the fasciculi of smooth muscles (Fig. 8).

We also observed a focal self-digestion in the smooth muscle fibers of the ripe cervices (Fig. 7). These muscle cells decreased in size and became a thin ribbon-like cell (see below). This was also observed in the histological sections. Autophagic vacuoles, autophagosomes, lysosomes, and enlarged clear spaces appeared in the vicinity of the myofilaments of smooth muscle cells. The presence of these clear spaces and autophagosomal vacuoles indicates the proteolytic activities within these cells. Significantly, apparent hydrolytic activities were also observed in the extracellular matrix. The clear halo spaces are suggestive of a dissolution of collagen fibers on the edge of the vesicles of phagosomes and lysosomes observed mostly within the proximity of the smooth muscle cells. These were found in the midst of collagen fibers and were surrounded by partially digested collagen and extracellular matrix components (Fig. 9).

These morphological findings suggest a possibility that collagen and other extracellular matrix components may be digested in part by the enzymes secreted by the autophagosomes or lysosomes of the smooth muscle cells.

**Atrophy of Smooth Muscle Cells in the Softening Cervices.** Following the hyperplastic and hypertrophic phase of pregnancy (5–12 days of pregnancy), the smooth muscle cells progressively decreased in number. Numerous smooth muscle cells became slender and had an elongated ribbon-like appearance when near term (Fig. 10). The findings of the electron microscopic study confirm our previous observation (44).

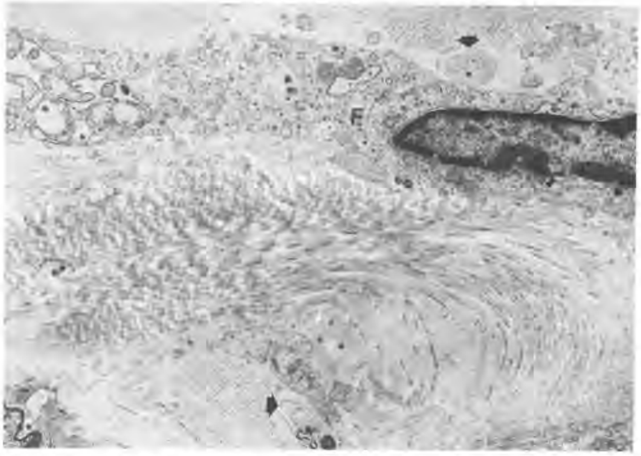


**Figure 9.** Electron micrograph of cervical tissues obtained from pregnant rat at 21 days of gestation, the same section as that in Figure 8 (×23,000). Fibroblasts (F), adjacent to the fasciculi of smooth muscle cells, appear to be in a stage of deterioration. The phagosomes/lysosomes (arrows) are conspicuous, numerous lysosomal vacuoles in the cytoplasm and the phagosomes/lysosomal vesicles in the periphery of fibroblasts are surrounded by clear spaces in the middle of the extracellular matrix. This suggests that enzymes released from the phagosomes/lysosomes vesicles might be responsible for digesting the surrounding structure.

## Discussion

In the present paper, we describe the orientation of collagen fibers and the direction of fiber formation as measured by the optical properties of the collagen fibers by polarized microscopy. The orientation of collagen fibers was demonstrated by a topo-optical reaction using picrosirius red F3BA dye, which has an elongated (4.5 nm) anionic molecule (47). This anionic dye binds through ionic interaction with the positively

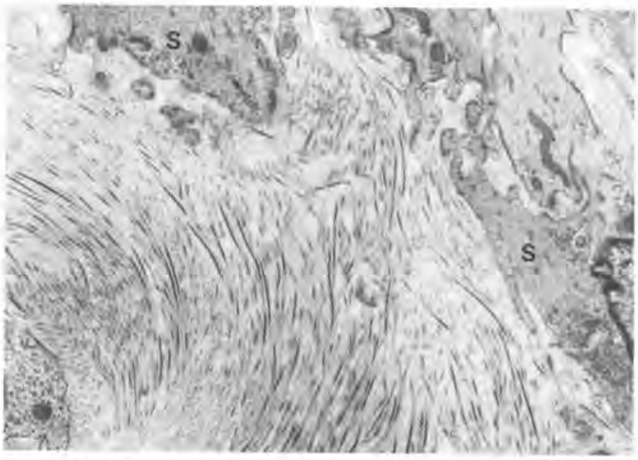


**Figure 8.** Electron micrograph of cervical tissues obtained from nonpregnant control rat (×23,000). Collagen fibers are well packed between the fasciculi of smooth muscle cells (S).

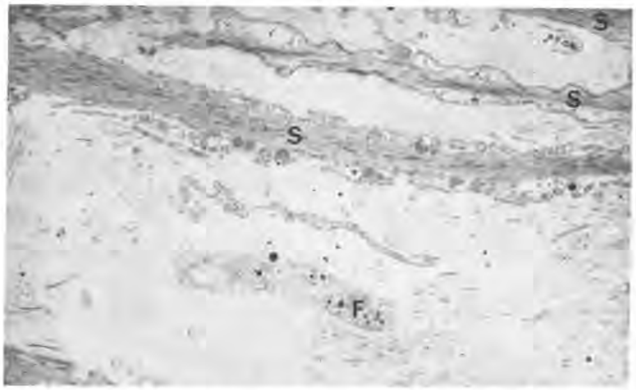


**Figure 10.** Electron micrograph of cervical tissues obtained from pregnant rat at 21 days of gestation (×17,000). Smooth muscle cells (S) have slender and elongated ribbon-like appearance. Clear spaces within the collagen structure are seen in the areas adjacent to these cells. A portion of fibroblast (F) is seen.

charged groups of collagen, probably lysine, hydroxylysine, and histidine moieties. When the fibrils of the collagen were arranged parallel to the long axis of the fibers, resulting in a sequential distribution of the positive charges on the surface of the collagen fibers, the dye molecules would bind parallel to the long axis of the organized collagen fibers. This resulted in induction and enhancement of birefringence of the fibers (48, 49).

Firm cervices, or those of nonpregnant rats, contained anisotropic collagen fibers. In contrast, the collagen fibers in the soft or dilated cervices were optically isotopic. The latter collagen fibers thus were most likely formed from either nonoriented collagen polymers or noncrystalline fibrils.

Normally, anisotropic fibers have different optical properties according to the position of polarized light, and show more than one refractive index (50, 51). Examples of anisotropic fibers are natural fibers such as silk or cotton. These natural fibers are intensively birefringent in respect to length of the fibers. Well-oriented collagen fibers show a similar positive birefringence in respect to the length of the fibers. Long polarized molecules of fibrillar collagen are aligned parallel to the long axis of the fibers (49, 50–52). This fibrous collagen arrangement is not only important to maintaining the structural integrity of the collagen structure, but also has a great impact on strengthening the tensile force of the collagen fiber (13, 25, 53, 54). By contrast, those fibers made up of an aggregate of small fibrils randomly oriented to each other show no birefringence (50).

These studies also provide information on the direction of the long axis of collagen or the formation of collagen fibers in the cervix. The disoriented and nonparallel collagen network showed a different birefringence color from the fibers oriented perpendicular to the plane of polarized light. All of those fibers oriented parallel to each other formed normal packed collagen structures, as noted in the nonpregnant cervix. These fibers all showed the same parallel bluish-green birefringence color ribbons when the fibers were placed parallel to the plane of polarized light.

Our findings confirm histological observations that collagen fibers in the ripe cervices are remodeled into dispersed fine fibers (44).

The finding of loss of orientation of collagen fibers in the ripening cervices suggest that these collagen fibers were disarranged. These changes probably account for the loss of their tensile strength due to the failure of fibrils to assemble into a parallel fibrous structure.

There is also a possibility that an excess amount of dermatan sulfate proteoglycans might coat the collagen fibrils and prevent interaction between collagen fibrils, since the molecules of proteoglycans have multiple anionic charge groups. Woessner et al (28–30) speculate that the excess amount of small dermatan sulfate proteoglycans in the ripening cervices may disperse collagen fibrils and lead to disorganization of the collagen fibers and softening of the cervices. Therefore, it is possible that the structural alteration of collagen fibers in the pregnant cervices may be in part due to a structural expansion in all dimensions (swelling of the structure without substantial increase of collagen mass) by increasing the concentrations of small molecular weight proteoglycans and water. This may result in opening the well-packed collagen fibrous structures and transforming them into the less-oriented collagen fibers.

The collagen concentration decreased precipitously as the tissue became soft and distensible. In order to examine the degradation of collagen in ripening cervices, studies were conducted to detect collagenase in pregnant animals (32, 33, 37–41). However, there was no significant collagenase activity found in the extracts of the cervices (30).

Recently, however, we were able to detect the turnover of collagen in the cervices of pregnant rats by an isotopic experiment (55). The collagen was labeled *in vivo* by injecting C-14-proline in the pregnant rats at 14, 15, and 16 days of gestation. The rats were sacrificed at 18 and 20 days. Total radioactivity in the insoluble collagen fractions in the cervices were studied. We found that the prelabeled insoluble collagen decreased during the period between 18 and 20 days, and it was estimated that a loss of 29% of the prelabeled collagen occurred over a 2-day period. In this experiment, we also found that the evidence of collagen synthesis in the tissues was accompanied by collagen turnover. Collagen synthesis was also supported by our previous findings, which showed elevated levels of mRNA for pro-a-1-[I]-collagen in the cervices of pregnant rats (12). It appears that the collagen remodels its structure in part by the processes of active degradation and synthesis.

Electron microscopy in this present study suggests that in the cervices of pregnant rats the enzymes associated with smooth muscle cells and fibroblasts may play a role in the degradation of collagen fibers. This process may occur through a combination of multiple proteolytic enzymes derived from lysosomal vesicles. In terms of the degradation of extracellular matrix, it has been reported that proteinases from white cells could slice collagen fibers into fragments extracellularly. These fragments subsequently are phagocytosed by connective tissue cells, and degraded within the cells (56). However, we observed that in the cervical tissues there appeared sporadic evidence suggestive of hydrolytic process of collagen fibers occurring mainly outside of the cells, since there was no evidence for resorption of collagen fibrils within the cells of fibroblasts or smooth muscle cells. Significantly, these smooth muscle cells and fibroblasts are in the process of dying (44), and we are suggesting that the apoptotic cells may plausibly contribute, in part, to the disruption of collagen structures by partial digestion of collagen fibers.

Thus, in summary, cervical softening in pregnancy occurs through a combination of both rearrangement of collagen fibers and the processes of collagen synthesis and degradation. The relative contribution of rearrangement, as opposed to synthesis and degradation, to cervical physiology in gestation has yet to be determined.


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