

Research Article

Structural Changes in Utrine Cervix of Female Albino Rat during Estrous Cycle and Pregnancy

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Abstract

Background: Many modifications occur in the uterine cervix during estrous cycle, pregnancy and postpartum in order to accommodate different functions

Aim of the work: is to study the histological changes occur in the rat uterine cervix during pregnancy and after delivery to explain its role in achieving successful pregnancy and labour.

Material and methods: This study was based on biopsies taken from the uterine cervix of 20 adult female albino rats randomly assigned into five groups (five animals per group): Group 1 estrous (non pregnant (NP)), group 2 at 15 days of pregnancy, group 3 at 18 days of pregnancy, group 4 at 22 days of pregnancy and Group 5 at 24 hours postpartum. Uterine cervix was taken out and processed for light microscopic, immunohistochemical and transmission electron microscopic studies.

Results: In the estrous rat cervix, the lining epithelium was columnar and thrown into endocervical glands. The greater proportion of the cervical wall was composed of dense fibrous connective tissue. On day 15 and 18 of pregnancy, less densely arranged collagen and high vascularity was noticed. On day 22 of pregnancy, evidence of tissue breakdown was apparent with large empty areas from collagen. Smaller empty areas and beginning of reorganization of collagen was seen 24 hours postpartum. In all physiological stages studied (estrous, pregnant and postpartum) fibroblastic cells of the cervical tissue showed different reaction to anti-desmin among groups but never reacted with anti- α -SMA antiserum.

Conclusion: During pregnancy extensive tissue remodelling involves extracellular matrix and cells of the cervical tissue. Collagen remodelling is a key event for ripening and delivery. Cross talking between fibroblast, mast cell, eosinophil and macrophage is important for collagen remodelling. Collagen remodelling can be modulated to achieve normal labor and avoid surgical delivery.

Keywords: Cervix, pregnancy, ripening .

Introduction

Many modifications occur in the uterine cervix during pregnancy. It acts as a barrier, retaining the fetus and preventing the entry of physical and microbiological agents that may affect the normal development of the fetus.

The functions of the uterine cervix change considerably during pregnancy. As the uterus enlarges to accommodate the growing fetus, the cervix behaves essentially as a barrier. At term, however, the cervix softens and dilates through a process known as cervical ripening. This process is extremely complex and involves interactions between different cellular compartments and the extracellular matrix, as well as properly timed biochemical cascades, and stromal infiltration by inflammatory cells

[1]. Since the main component of the uterine cervix is connective tissue, collagen remodeling is a key event for ripening and delivery⁽¹⁾.

Cervical ripening is an energy-dependent process that requires an adequate supply of nutrients. The vascular system and new vessel formation (angiogenesis) are critical for the cervical histo-architectural changes that are necessary for a successful vaginal delivery. Although angiogenesis is essentially an endothelial cell event, other cell types and various mediators are involved in this process. Studies in vitro and in vivo have implicated mast cells in angiogenesis⁽²⁾. Fibroblasts play an important role in cervical functions. Changes in the cytoskeletal elements are prominent features in the morphological alterations in

fibroblasts with desmin and α -smooth muscle actin (α -SMA) frequently being expressed in specific pathways of differentiation⁽⁷⁾.

The aim of this work is to study the histological changes occur in the rat uterine cervix during pregnancy and after delivery to explain its role in achieving successful pregnancy and labour.

Material and methods

Animals: This study was based on biopsies taken from the uterine cervix of 20 adult female Sprague Dawley rats. The animals aged approximately 12 weeks old and weight 220 to 250 gm. To obtain pregnant specimens, pro-estrous females were caged overnight with males of proven fertility. Day 1 of pregnancy (D1) was defined as the presence of spermatozoa in the vaginal smear⁽⁸⁾. In our colony, delivery occurred on D22. Rats were randomly assigned to each of the different experimental groups (five animals per group): Group I: Estrous (non pregnant NP), group II: at day 14 of pregnancy, group III: at 18 days of pregnancy, group IV: at day 22 of pregnancy Group V: at 24 hours after delivery

At the end of the experiment, the rats were sacrificed by decapitation under light halothane anesthesia, specimen of cervix were removed, For light microscopy, the specimens were fixed in 10% neutral-buffered formalin, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin blocks, then specimens were washed by tap water and processed to prepare tissue sections for histological, histochemical and immunohistochemical study.

1. Light microscopic study

Histological sections of 5um thickness were stained with haematoxylin and eosin stain and masson trichrome⁽⁹⁾.

2. Ultrastructural study

For transmission electron microscopy, the specimens were fixed for 2 hours in 2.5% phosphate buffered glutaraldehyde solution (pH 7.4) at 4°C. After washing with phosphate buffer, the specimens were post-fixed for 1 h in 1% buffered osmium tetroxide solution. Subsequently, the specimens were dehydrated through an ascending series of ethanol, treated with propylene oxide and embedded in epoxy resin. After heat polymerization, sections were cut using an ultramicrotome with a diamond

knife and were double-stained with uranyl acetate and lead citrate to be examined by a JEOL electron microscope at EM unit, Assuit University⁽¹⁾.

Immunohistochemical techniques:

Immunocytochemical staining was performed using mouse monoclonal antibodies (anti- Anti desmin antibody and Anti smooth muscle actin antibody) which were obtained from sigma aldrich. Sections were deparaffinized, hydrated then washed in 0.1M phosphate buffer saline (PBS). Endogenous peroxidases were quenched by treatment with H₂O₂ in methanol (Peroxidase blocking solution) followed by washing in tris buffer saline (TBS). Non-specific binding of IgG was blocked using normal goat serum. The sections were incubated with the diluted primary antibodies for overnight at room temperature. Sections then were washed 3 times each in buffer and incubated for further 30 minutes with biotinylated goat anti-rabbit secondary antibodies. Following further 30 minutes incubation with Vectastain ABC kits (Avidin, Biotinylated horse radish peroxidase Complex) and washing for 10 minutes, the substrate, diaminobenzidine tetra hydrochloride (DAB) in distilled water was added for 5-10 min. The enzyme reaction was developed as described previously. The slides were lightly counterstained by hematoxylin. This substrate gives brown color at the immunoreactive sites⁽¹⁰⁾.

Photography: Olympus light microscopy was used. Slides were photographed using Olympus digital camera. Images were processed using Adobe Photoshop.

Results

A) Histological study of the non pregnant estrous uterine cervix:

The lining epithelium was columnar (Fig.1). The subepithelial connective tissue was vascular with many eosinophils. The greater proportion of the cervical wall was composed of dense fibrous connective tissue consisting of compactly and regularly arranged collagen fibers with fibroblasts and eosinophils embedded in sparse ground substance. The smooth muscle fibers formed an incomplete muscularis in the middle and deeper layers of the cervical wall (Figs. 1,2). Blood vessels were present throughout the depth of the tissue, but were most numerous in

the deepest layers (Fig. 5). The compactly and regularly arranged collagen fibers taking the blue colour in Masson trichrome stained sections (Fig. 6). The endocervical glands were well observed in toluidine blue stained semithin sections. A variety of connective tissue cells including mast cells, neutrophils and eosinophils were also observed (Fig. 7). With TEM the bundles of collagen fibrils were closely packed with relatively little intervening ground substance while the fibrocytes possessed a high nucleus to- cytoplasm ratio and generally looked inactive, the majority lacking long cytoplasmic processes, well-developed granular reticulum, and the cell body contained an oval or elongated nucleus and a variable number of sparsely distributed organelles (Fig. 8).

B) Histological study of the pregnant and postpartum uterine cervix:

1) Light Microscopic Study:

On day 15 of pregnancy, there was marked increase in the vascularity of the cervical wall. There was a complete network of subepithelial capillaries with a marked increase in the size of the vessels in the outer part of the cervical wall (Figs. 9 & 10). The rich vascularization was the main finding with budding of new vessels from resident ones as an evidence of angiogenesis (Fig. 11). Less densely arranged collagen which was associated with the exposure of smooth muscle fibers was clearly observed in Masson trichrome stained sections (Fig. 12). The subepithelial connective tissue showed evidence of tissue breakdown in the form of wide intercellular spaces with rich vascularization, on day 18 (Figs. 13 & 14) and 22 of pregnancy (Figs. 15 & 16). Neutrophils and Mast cell were more numerously observed on day 18 of pregnancy (Fig. 17), while widely distributed macrophages showing engulfed material were obviously observed On day 22 of pregnancy (Fig. 18). At 24 hours postpartum, the inflammation was still present and smaller "empty" areas from collagen were seen (Figs. 19 & 20) and it appeared that the collagen fibrils were beginning to reorganize (Fig. 21).

2) Ultrastructural study:

On day 15 of pregnancy, the connective tissue was mainly comprised of bundles of collagen fibrils orientated together with small inactive fibrocytes, collagen breakdown started to occur at this age of pregnancy (Figs. 22).

In contrast, the cervical connective tissue in late pregnant rat (days 18 & 22 of pregnancy) exhibited bundles of collagen fibrils widely separated by pale-stained ground substance. Large, star shaped active looking fibroblasts; with an oval nucleus, a well-developed granular reticulum, numerous small mitochondria and few pinocytosis vesicles were observed (Fig. 23). Eosinophils usually surrounded by empty areas from collagen (Figs. 24 & 25). Some eosinophils showed inverted density core granules with the light zone in the center of the granule and presence of tubulovesicular structures were observed (Fig. 26). Macrophages with mega lysosomes were also an important finding during late pregnancy (Figs. 27).

Smaller empty areas of collagen were seen with still few active- looking fibroblasts 24 hours after labour (Fig. 28).

Expression of desmin and α -smooth muscle actin in cervical Fibroblastic Cells:

In all physiological stages studied (estrous, pregnant and postpartum) fibroblastic cells of the cervical tissue were always positively stained with anti-desmin but never reacted with anti- α -SMA antiserum (Figs. 29 & 30). Desmin intensity varied dramatically during pregnancy. Faint expression pattern for desmin noticed in the estrous, 15 days and postpartum stages. High expression was noticed at 18 days. Maximal expression achieved at 22 days of pregnancy. Staining pattern for desmin corresponds to the area surrounding the nucleus then radiating through the processes at the maximal level (Fig. 31)



Figure 1. The cervix of non pregnant estrous rat showing the simple columnar epithelial lining (arrow), connective tissue stroma (star) and blood vessel (b.v.) H&E, X 100.

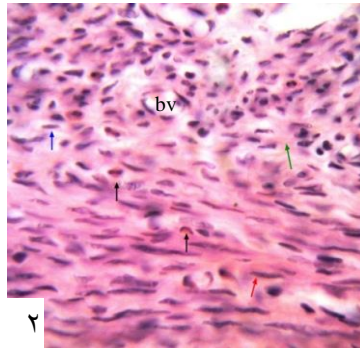


Figure 2. Higher magnification of figure 1 showing eosinophils (black arrow), fibroblasts (blue arrows), smooth muscle fibers (red arrow), collagen fibers in between (green arrow) and blood vessel (bv) . H&E, X 400.

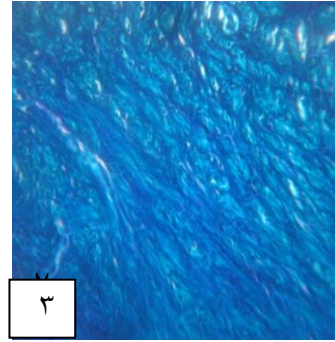


Figure 3. Section of the cervix of non pregnant showing the compactly and regularly arranged collagen fibers. Masson trichrome, X 1000.

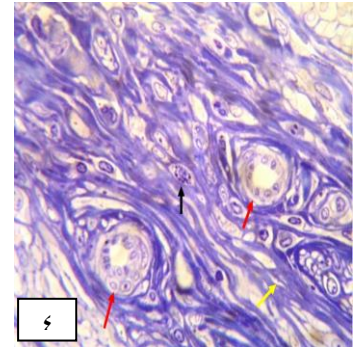


Figure 4. Semithin section of the cervix of non pregnant estrous rat stained with toluidine blue showing regularly arranged collagen (yellow arrow), cervical glands (red arrows) and mast cell (black arrow), X 1000.

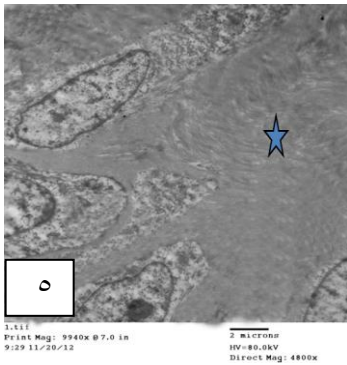


Figure 5. An electron micrograph of non pregnant estrous rat cervix showing multiple fusiform fibrocytes and dense stroma (star) X 9940.

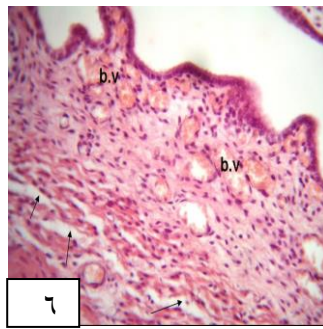
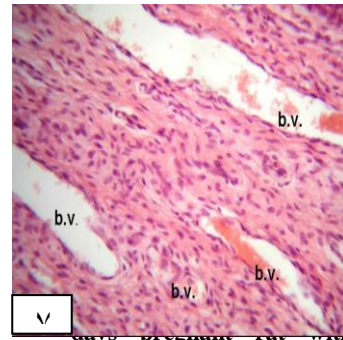


Figure 6. The uterine cervix of 14 days pregnant rat showing rich vascularization and the less densely arranged collagen (arrows). blood vessels (b.v.). H&E, X 400.



markedly dilated blood vessel (b.v.). H&E, X 400.



budding of new blood vessels (arrow). H&E, X 1000.

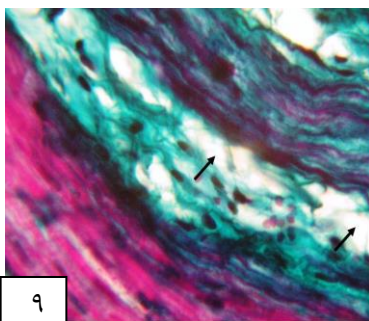


Figure 9. The cervical stroma of 14 days pregnant rat showing exposure of smooth muscle fibers (red) and collagen empty areas (arrows). Masson trichrome, X 1000.



Figure 10. The cervical stroma (s.) of 14 days pregnant rat showing rich vascularization (arrows) H&E, X 400.

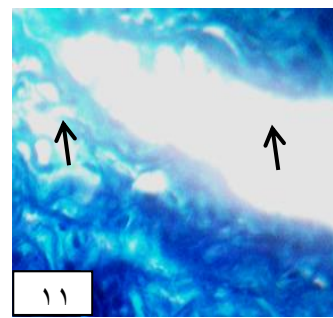


Figure 11. The cervical stroma of 14 days pregnant rat showing wide empty areas (arrows). Masson trichrome, X 1000.

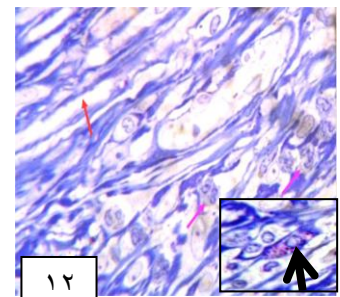


Figure 12. Semithin section of the cervix of 14 days pregnant rat stained with toluidine blue showing neutrophils (pink arrows) and wide separated collagen fibers (red arrows). Notice mast cell with meta-chromatic granules (inset) X 1000.

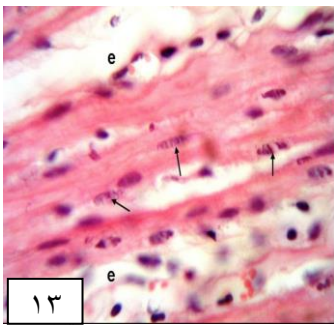


Figure 13. The cervical stroma of 22 days pregnant rat with large spaces separating collagen bundles (e) and many spindle shaped fibroblasts (arrows). H&E, X 1000.

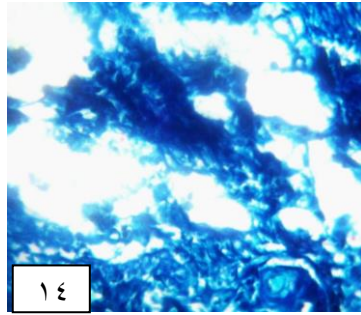


Figure 14. The cervical stroma of 22 days pregnant rat showing marked collagen breakdown (arrows). Masson trichrome, X 1000.

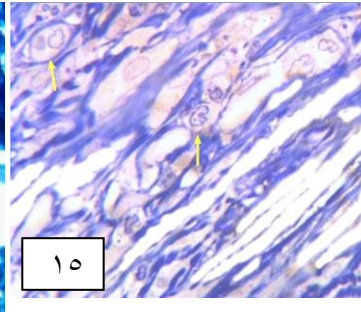


Figure 15. Semithin section of the cervix of 22 days pregnant rat showing macrophages (arrows), smaller empty spaces (blue arrow). H&E, X 1000.

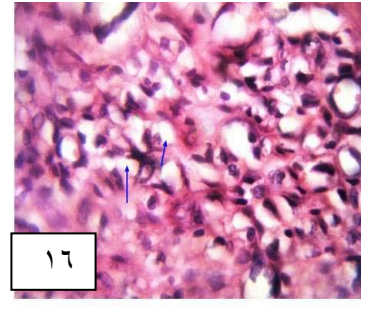


Figure 16. The uterine cervix 24 hours after labor showing inflammatory cell infiltration (blue arrow). H&E, X 1000.

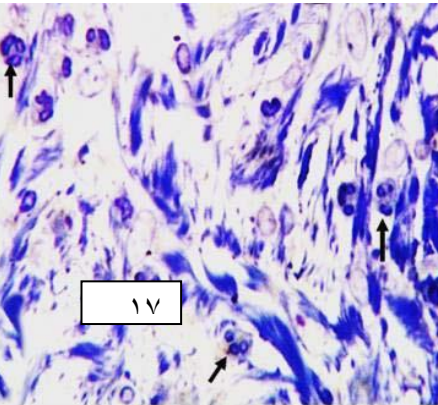


Figure 17. The uterine cervix 24 hours after labor showing blue staining and inflammatory cell infiltration (black arrows), X 1000.

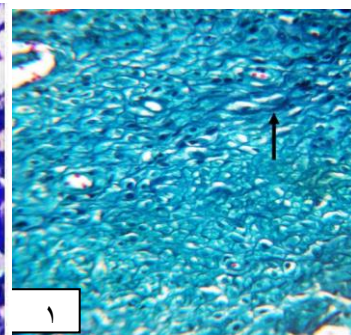


Figure 18. The uterine cervix 24 hours after labor showing that collagen fibers began to restore their normal arrangement (arrows). Masson trichrome, X 4000.

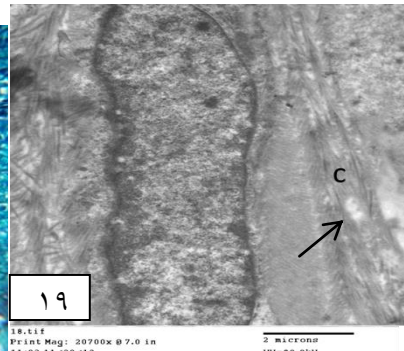


Figure 19. An electron micrograph of 14 days pregnant rat cervix showing fibrocytes and collagen breakdown (arrow) X 20000.

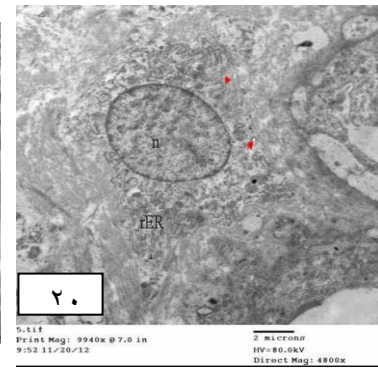


Figure 20. An electron micrograph of 18 days pregnant rat cervix showing a fibroblast with increased rER, pinocytotic vesicles (arrowheads) and round to oval euchromatic nucleus (n) X 99400.

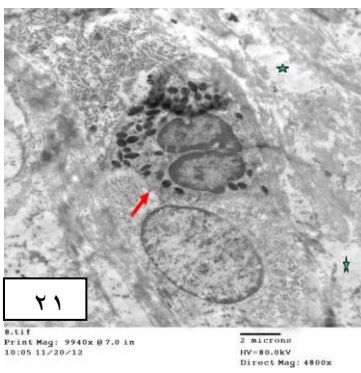


Figure 21. An electron micrograph of 18 days pregnant rat cervix showing an eosinophil (arrow) surrounded by collagen empty areas (stars), X 99400.

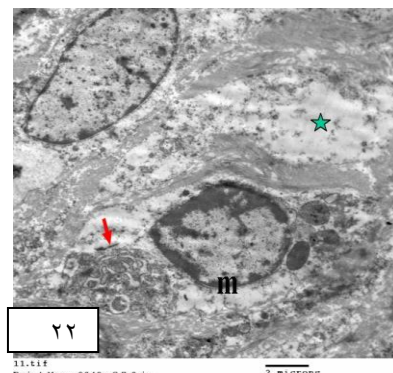


Figure 22. An electron micrograph of 18 days pregnant rat cervix showing a macrophage (m) with a mega lysosome (arrow) and evident collagen breakdown (star), X 99400.

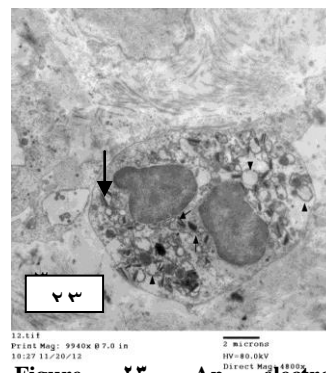


Figure 23. An electron micrograph of 22 days pregnant rat cervix showing an activated eosinophil with the electron dense part towards the periphery of some granules (arrows) and tubulovesicular structures (arrow heads). Notice area of collagen depolymerization (P) X 99400.

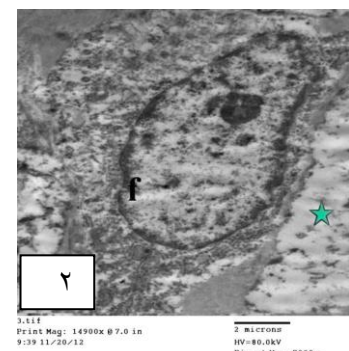


Figure 24. An electron micrograph of rat cervix 24 hours after labour showing a fibroblast (f) and smaller collagen free areas (star), X 149000.

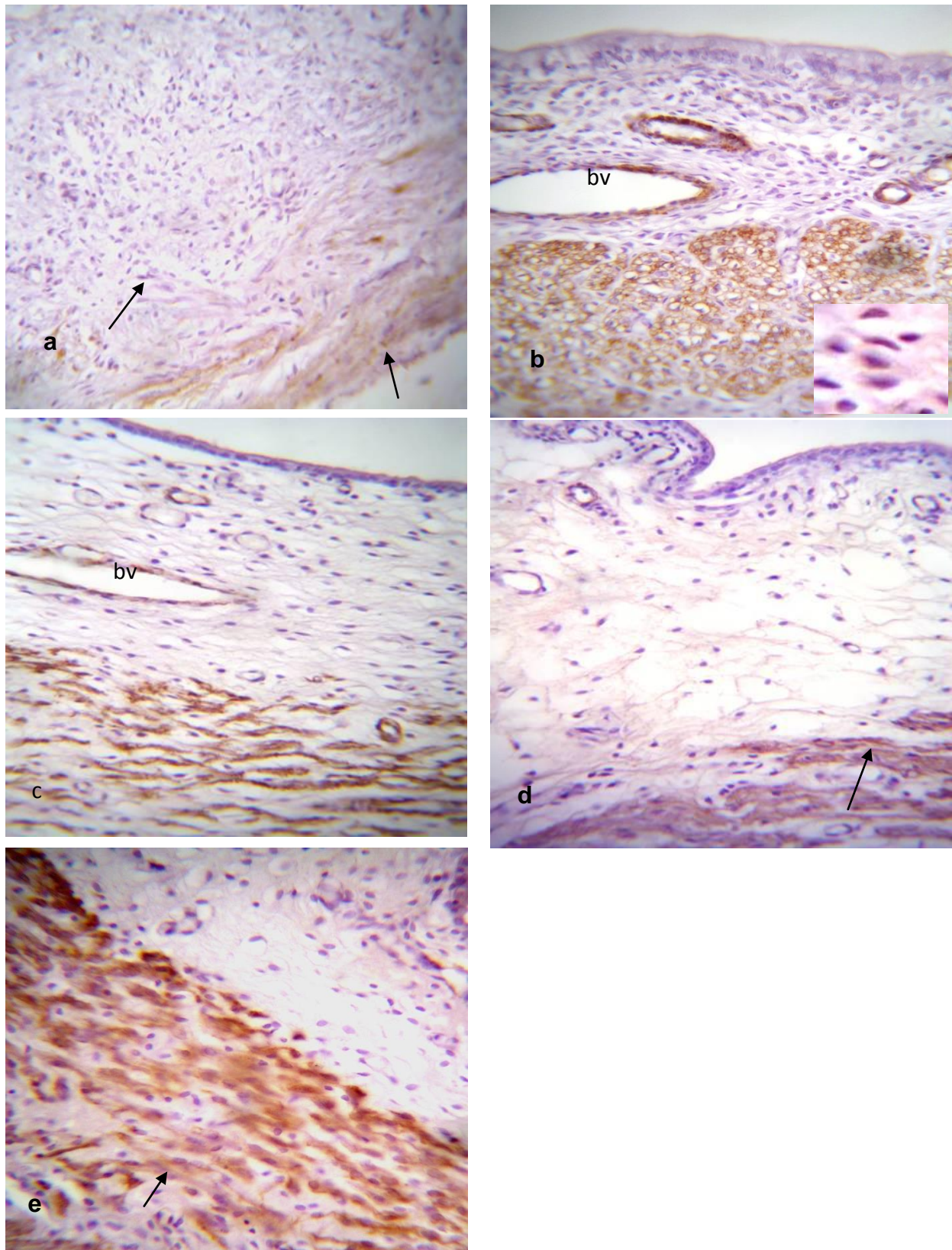


Figure 20. A photomicrograph of estrous (a), 14 days pregnant rat (b) 18 days pregnant rat (c), 22 days pregnant rat (d) and 24 hours postpartum (e) rat cervix showing negative stromal fibroblastic α -SMA expression (inset) in comparison to smooth muscle cells (arrows). Notice positive expression in the blood vessels (bv). Anti α -SMA immunolabelled sections, X 400. Inset X1000.

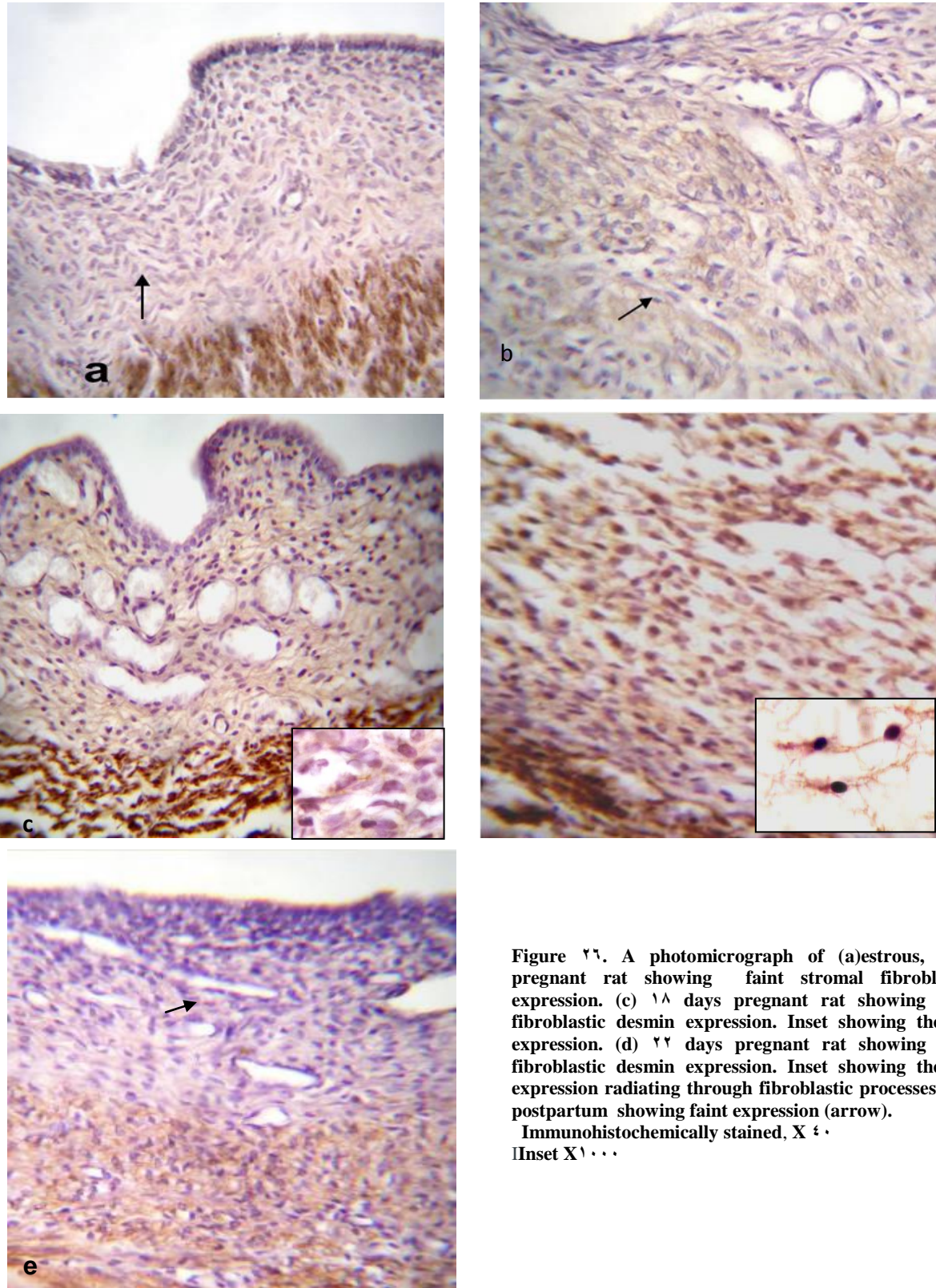


Figure 26. A photomicrograph of (a)estrous, (b) 14 days pregnant rat showing faint stromal fibroblastic desmin expression. (c) 18 days pregnant rat showing high stromal fibroblastic desmin expression. Inset showing the cytoplasmic expression. (d) 22 days pregnant rat showing high stromal fibroblastic desmin expression. Inset showing the cytoplasmic expression radiating through fibroblastic processes. (e) 24 hours postpartum showing faint expression (arrow).
Immunohistochemically stained, X 400
Inset X 1000

Discussion

Cervix has an important role in the normal transport and capacitation of spermatozoa, as well as acting as a protective barrier. The cervix also serves to prevent the expulsion of the preterm conceptus. At term, however, the cervix softens and dilates through a process known as cervical ripening⁽¹⁾. It is a requirement for successful labor that there is adequate cervical softening combined with effective myometrial contractions. This is accomplished by an extensive remodeling of the extracellular matrix in both the cervix and the uterus, executed by different types of cells and mediators in a complex interplay⁽²⁾. Many factors including matrix metalloproteinases (MMPs) and extracellular matrix (ECM) components are known to regulate this remodeling⁽³⁾. This study aimed at the investigation of the histological changes that occur in rat uterine cervix during different stages of gestation in comparison to the non pregnant estrous cervix and the postpartum. The work investigated also the expression of the antibodies against desmin, and α -smooth muscle actin (α -SMA) in cervical fibroblasts.

Histologically, by 14, 18 & 22 days gestation there was evidence of tissue breakdown, rich vasculature and inflammatory cells infiltration. Active collagenolysis occurs during pregnancy. This might be the underlying mechanism of cervical softening. Final cervical ripening, collagenolysis and inflammation are closely related processes; Granulocytes were surrounded by a halo of degraded collagen. The presence of chemokines, such as interleukin-1 (IL-1) is an important stimulator of granulocyte invasion^(4,5). Additionally, it has been reported that other pro-inflammatory cytokines, such as IL-1, tumor necrosis factor (TNF) and IL-6, are secreted within the cervix at parturition⁽⁶⁾. It has also been hypothesized that a decrease in progesterone levels and leakage of cytokines or prostaglandins from the fetal membranes, leads to an increased expression of IL-1 and the onset of an inflammatory cascade within the cervix⁽⁷⁾. Inflammatory cells invading the cervix towards late gestation provide a potential source of collagenase and neutral proteinase activity^(8,9).

In the present study, there was marked increase in the vasculature of the cervix in mid and late pregnancy. Mast cells were markedly observed in most ages studied. Mast cells (MCs), which have been shown to accumulate around vessels and new capillary sprouting sites, might be implicated in angiogenesis. In agreement with this study Varayoud et al.,⁽¹⁰⁾ observed mast cells (MCs) greatly during mid-pregnancy. They also observed rich vasculature and new vessel sprouting observed in mid and late pregnancy. It has been demonstrated that histamine and heparin have potent angiogenic effects. The complex phenomenon of angiogenesis begins with degradation of the basement membrane by cellular proteases, allowing the endothelial cells to penetrate and migrate into the extracellular matrix and then Proliferate⁽¹¹⁾. Recent studies reveal that many factors, including growth factors and integrins, regulate the process of angiogenesis⁽¹²⁾. Uterine MCs release several cytokines, growth factors and chemokines that are crucial for stimulating inflammation^(13,14). Therefore, it is highly possible that MCs may be key contributors to uterine "inflammatory-state", during human parturition^(15,16). Finally, studies suggest an effect of corticotrophin releasing hormone (CRH) on uterine MC activation as a mechanism to evoke uterine contractions⁽¹⁷⁾, whereas the relaxant hormones, relaxin and progesterone, are believed to inhibit uterine MC activation^(18,19).

The extracellular matrix of the uterine cervix is composed mainly of collagen (types I and III), elastic fibers and proteoglycans⁽²⁰⁾. It has been demonstrated that activated MCs have the potential to stimulate the production of matrix metalloproteinases (MMPs) by endometrial stromal cells and to set up a cascade of MMP activation within the endometrium⁽²¹⁾. The MMP system is comprised of the proteolytic factors, the MMPs, and their tissue inhibitors (TIMPs)⁽²²⁾. Zhang and Warren⁽²³⁾, added that collagenases (MMP-1, -8 and -13) cleave both fibrillar and non-fibrillar collagens⁽²⁴⁾.

Mast cells in the uterus, co-express MMP-1 and mast cell tryptase⁽²⁵⁾. Reports have implicated both MCs tryptase and chymase in the activation of precursor forms of the matrix

metalloproteinases⁽⁷⁾. The delicate balance between the activity of the MMPs and their inhibitors plays the key role for the normal tissue growth and remodeling^(7,8). It was detected that in women with a successful cerclage placement, MMP-1 and MMP-8 were not detected over the study time period which is indicative of their role in tissue degradation and cervical ripening⁽⁷⁾.

MC could profoundly affect the various morphological changes associated with pregnancy and labour. These were in contrast to Menzies et al.,⁽⁷⁾ who suggested that mast cells do not have a vital role in the induction of labor, or in the promotion of labor-associated inflammation.

In the present study, the presence of mast cells was often associated with the influx of inflammatory cells such as eosinophils, neutrophils and macrophages. Although many aspects of eosinophil function remain to be elucidated, eosinophils are known to elaborate inflammatory mediators that can potentially bring about tissue damage. Such factors include the major basic protein⁽⁷⁾.

Norrby and Woolley⁽⁷⁾, found many factors released by activated MCs might explain the accumulation of eosinophils and neutrophils in the endometrium at menstruation. Whatever the specific MC-eosinophil-neutrophil interactions may be, it seems clear from this histological study that these three granulocytes assume important functional roles in relation to the tissue and vascular remodeling associated with pregnancy. Eosinophils may regulate monocyte differentiation and macrophage polarization during postpartum repair⁽⁷⁾.

In the present study, macrophages were observed in most cervical stages. Accumulating evidence suggests the involvement of uterine macrophages in a wide range of gestational process. Macrophages support vascular remodeling by removing apoptotic cells and producing pro-angiogenic factors⁽⁷⁾.

In this study, the ascending expression of desmin towards the end of pregnancy is in agreement with Varayoud et al.,⁽¹⁾ who explains

that this might contribute to the cellular compartment adaptation to the changes of the regional forces produced by alterations in the ECM composition.

An important aspect of the differential expression of desmin is its mechanism of control. It is interesting to mention that desmin expression increased in parallel with the plasma levels of relaxin described for pregnant rats. Relaxin reaches significant plasma levels from D¹⁰ of pregnancy showing a significant elevation 2-3 h before parturition. Desmin increased its expression from D¹⁰, and the highest levels of expression are reached just before parturition. Moreover, immediately after delivery, both relaxin and desmin reach basal levels⁽⁷⁾. In this study fibroblast never reacts with anti- α -SMA antiserum, so it could be not developed to myofibroblast. In contrast Andrea et al.,⁽⁷⁾ found that Cultured human myometrial cells expressed smooth muscle actin and fibroblast markers (vimentin, β), suggesting that they may represent an intermediate myofibroblast phenotype.

Fibroblasts differentiated and showed increased secretory characteristics, at the ultrastructural level. In the early pregnancy, they were cylindrical lacking a well-developed granular reticulum, long cytoplasmic processes. While in late pregnancy they assumed many cytoplasmic processes and well developed granular reticulum. Desmin expression in fibroblasts could account for the rapid transformation of cell shape and organelle distribution as a consequence of increased synthetic activity of collagen and elastin fibers during pregnancy. Differential expression of desmin and the electron microscopic observations, modulate fibroblastic phenotype in the uterine cervix during pregnancy and suggest a secretory role for these cells⁽⁷⁾.

It can be concluded that during pregnancy extensive tissue remodeling involves both the extracellular matrix and cells of the cervical tissue. Collagen remodeling is a key event for ripening and delivery. Cross talking between fibroblast, mast cell, eosinophil and macrophage is important for collagen remodeling. By understanding these changes, collagen remodelling can be modulated to achieve normal labor and avoid surgical delivery.

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