# The bovine cervix explored:

## The cow as a model for studies on functional changes in the uterine cervix.

**Vidya N.A. Breeveld-Dwarkasing**

*Cover:*
*"...at some point in time the softened cervix is stretched open under the influence of uterine contractions, to form a passageway for the fullgrown foetus into the outerworld".(Endoscopic picture of the dilating cervix of a cow in parturition)*

**CIP-gegevens Koninklijke Bibliotheek, Den Haag**

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The bovine cervix explored: The cow as a model for studies on functional changes in the uterine cervix/ Vidya Natasia Angelique Breeveld-Dwarkasing, Universiteit Utrecht, Faculteit der Diergeneeskunde.
Proefschrift Universiteit Utrecht. Met samenvatting in het Nederlands.
ISBN: 90-393-3026-3
Trefwoorden: runderen, cervix, partus.

Layout en Ontwerp omslag: Vidya Breeveld-Dwarkasing
Uitvoering omslag: Harry Otter en Henk Halsema
Druk: Addix, Wijk bij Duurstede

# The Bovine Cervix Explored.

## The cow as a model for studies on functional changes in the cervix uteri.

## Onderzoek naar de mogelijkheden voor het rund als modeldier in studies naar functionele veranderingen in de cervix uteri.

(met een samenvatting in het Nederlands)

**Proefschrift**

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 14 mei 2002 des ochtends te 10.30 uur

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Geboren op 13 maart 1966, te Paramaribo

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Het onderzoek beschreven in dit proefschrift is financieel mogelijk gemaakt door de Hoofdafdeling Gezondheidszorg Landbouwhuisdieren van de Faculteit der Diergeneeskunde.

*Dedicated to: the Foremost High*

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# General Introduction

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## Introduction

The dramatic change in structure of the cervix uteri during pregnancy and parturition has never ceased to intrigue researchers in the course of time. While the foetus is maintained in the safe, closed uterine environment during its development, at some point in time the softened cervix is stretched open under the influence of uterine contractions, to form a passageway for the full grown foetus into the outer world. The cervix is a firm, thick walled tube, lined with a mucus-secreting epithelium, connecting the uterus and the vagina. Since Danfort (1947) first reported that the cervix is a fibrous, collagen rich structure, today's advances in biochemistry and molecular biology have unravelled many aspects of cervical functioning leaving us, however, with many more questions to answer. The following overview by no means claimes to be complete. It only serves to introduce some of the many factors that are involved in the functional changes which occur in the cervix uteri during different stages of the reproductive cycle, including pregnancy and parturition.

Not only in the case of a pregnancy, but in many species also during the oestrous cycle, the cervix becomes softer during the follicular, oestrogen dominated phase (Hafez, 1973; Conrad and Hoover, 1982; Silva *et al*., 1995), while it remains firm during the progesterone dominated phase of the cycle.

An understanding of the regulation of the functional changes that occur in the cervix during these two distinct stages of the reproductive cycle, may be of great benefit to the diagnosis and management of several gynaecological and obstetrical problems in animal breeding and human health care. For example, it may be desirable to alter the properties of the cervix to enable penetration during artificial insemination or non-surgical embryo transfer in many species, including sheep (Khalifa *et al*., 1992) and goats (Pereira *et al*., 1998). Also, in the case of early (therapeutic) abortions in women there is a need to penetrate the cervix more easily (Greer *et al*., 1992; Bokström and Norström, 1995). Irreversible infertility in sheep occurs after prolonged exposure to phytoestrogens, because morphologic alterations of the glandular structures of the cervix occur which negatively influences the transport of spermatozoa (Adams, 1995), probably because the secretion products of the glands have also been changed.

Clinical problems during parturition occur when the cervix fails to dilate sufficiently or fails to dilate at all, as is the case in women with an unfavourable cervix (Granström *et al*., 1991; Stempel *et al*., 1997) or in sheep with ringwomb (Dobson, 1988). Primary causes of insufficient cervical dilatation are more difficult to diagnose, because they most likely originate from a disturbed regulation of either cellular or biochemical processes in the cervix. They need to be distinguished from secondary causes like uterine inertia, such as occurs during hypocalcemia in cattle or in the case of slow progressing labour such as with a too large foetus or an abnormal foetal presentation. Primary causes prompt for a caesarean section, whereas secondary causes may not always need surgical intervention.

In women, cervical incompetence leading to premature labour is considered to be a major cause of perinatal mortality (Avis *et al*., 1996; Garfield *et*

*al*., 1998). In addition, slow progress of spontaneous or induced labour due to a insufficiently dilating cervix may also lead to foetal distress and cause most of the damage to the foetus.

## Gross- and microscopical morphology of the cervix

The following general description is mainly based on Hafez (1973), Dobson (1988) and our own macroscopic and microscopic observations, part of which has been published in Breeveld-Dwarkasing *et al*. (2000).

Between species, there is great variation in the gross morphology of the cervix, most probably reflecting the different physiological mechanisms of sperm transport and parturition (Hafez, 1973). The cervical canal may be either straight or it may contain annular folds or rings of mucosa, which project into the lumen. There may be a single or a double canal (*e.g*. in rats). The cervix can project into the vagina with a clearly recognizable external os, or just gradually merge into the vaginal wall. The cervices of different species have more or less in common, however, that the mucosal and submucosal layers are of a fibrous nature and that collagen is the major constituent of the connective tissue in these layers. Together, these two layers are commonly referred to as the stromal layer.

Several tissue layers and cellular components can be distinguished in the cervix of all mammalian species:

1. The cervical canal is lined by a mucus secreting columnar epithelium, which may project into the stromal layer, forming more or less extensively branched glandular structures.
2. The mucosa has folds projecting into the lumen, which may be rather extensive in some species.
3. It has also been reported for some species (*e.g*. sheep: Fitzpatrick and Dobson, 1979; women; Scoutt *et al*., 1993; cows: our own observations) that the subepithelial part of the stromal layer can be distinguished from the deeper part, by a different cellular composition and/ or a softer texture.
4. The deeper part of the stromal layer contains many smooth muscle cells, which are unevenly distributed through the stroma.
5. However, the outer layer, lining the stromal layer is formed by a more defined smooth muscle layer, which is grossly divided into an inner layer, of more circularly arranged bundles and an outer layer of more longitudinally oriented bundles.
6. Dorsally and ventrocranially the cervix is covered by peritoneum.

The bovine cervix is a relatively muscular cervix (Fig. 1A-D). The outer smooth muscle layer is rather thick compared to that in other species. At the vaginal os, the muscle layer is relatively thin compared to the stromal layer, while at the uterine side of the cervix the muscle layer has become much thicker, as illustrated by figures 1C-D. The cervical mucosa forms 3-4 annular folds that

project into the lumen, but it also forms numerous smaller longitudinal folds (Fig. 1A). The stromal layer shows a clear histological demarcation into a subepithelial and a more cell dense deeper layer (Fig. 2). The deep stromal layer can also be distinguished from the subepithelial stromal layer by the presence of smooth muscle cells, interspersed between the fibroblasts. In the subepithelial layer smooth muscle cells are mainly of vascular origin (Fig. 3).

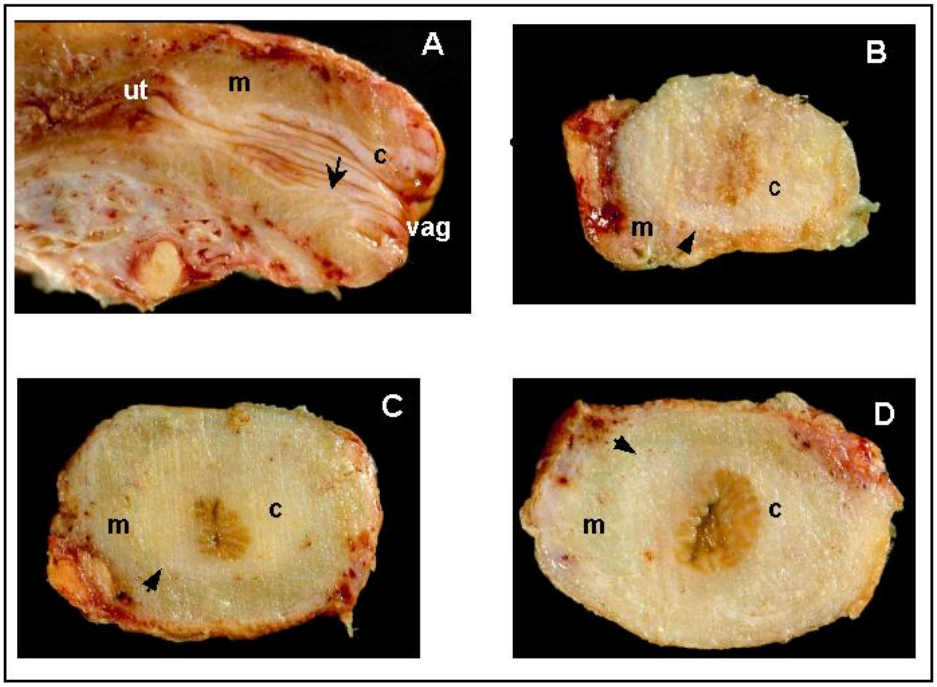


Fig. 1. Macroscopic morphology of the bovine cervix. **A**) longitudinal section: **vag** = vaginal part, **ut** = uterus, the long arrow points to one of the annular folds. Cross-sections at **B**) vaginal; **C**) mid and **D**) uterine level The short arrows indicate the border between t connective tissue layer (**c**) and the outer smooth muscle layer (**m**). (*Breeveld-Dwarkasing et al., 2000).*

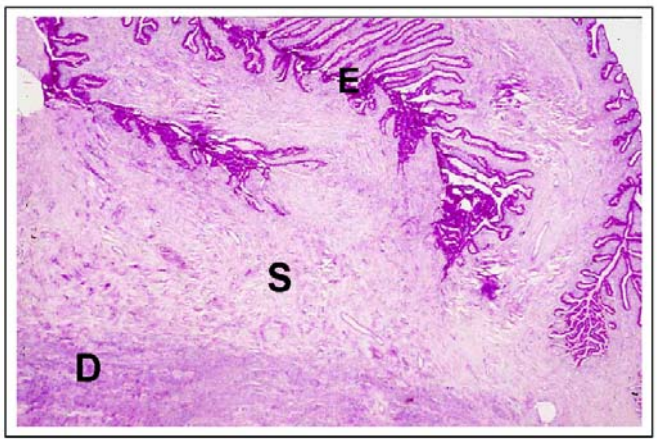


Fig. 2. Photomicrograph of a PAS-stained cross-section of the bovine cervix. A sharp demarcation between superficial stromal layer (**S**) and deep stromal layer (**D**) is clearly visible, **E** is the epithelial layer. Magnification: 400 ×.

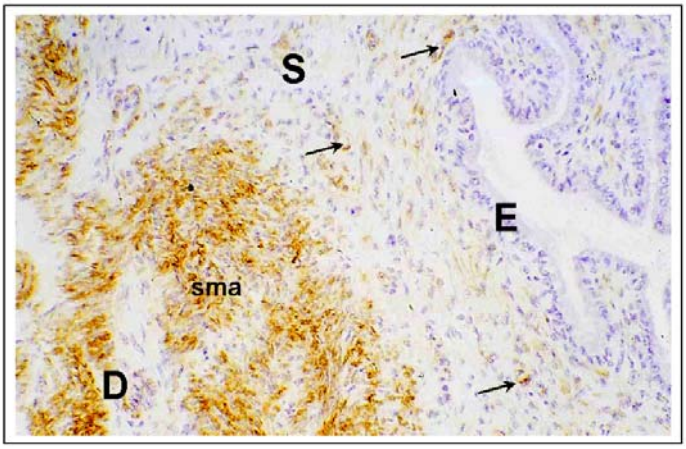


Fig. 3. Immuno histochemical localisation of smooth muscle actin (**sma**) in the bovine cervix. Please note that **sma** is almost exclusively located in the deep stromal layer (**D**) and that the scarce immunoreactivity in the superficial layer (**S**) is located around small blood vessels (**arrows**), **E** = epithelial layer (*Breeveld-Dwarkasing et al., 2000).*

## Cervical softening during the oestrous cycle

Softening of the cervix during the oestrous phase has been reported for several species (Hafez, 1973; Silva *et al*., 1995). At least for the cow, there are a few studies dealing with factors that might be involved in cervical softening during

the oestrous phase. During the pro-oestrous/oestrous period, increases in oxytocine receptors (Fuchs *et al*., 1996) and FSH receptors (Mizrachi and Shemesh, 1999) occur in the cervix, both of which can mediate an increase in cervical $PGE_2$ production. Locally applied $PGE_2$ induces cervical softening and opening in non-pregnant (Duchens, *et al*., 1993) and early pregnant cows (Lavoir and Betteridge, 1996).

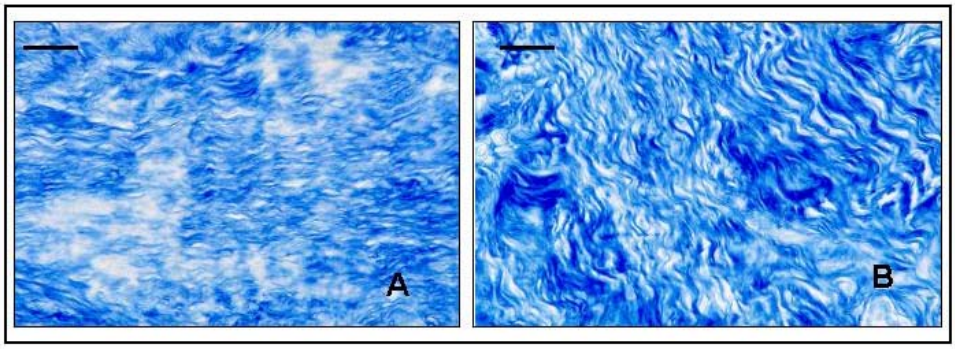


Fig. 4. Deep stromal layer of the bovine cervix, stained for collagen fibres. Note the less densely packed, shorter fibre length in an animal with low progesterone status (**A**), compared to a cow with high plasma progesterone concentrations (**B**). Scale bars represent 4μm.

In non-pregnant cows without a functional corpus luteum, we observed numerous areas of the deep stromal layer in which the collagen fibres were shorter and less dense, in contrast to cows that had a functional corpus luteum (Fig. 4A-B). This unpublished observation suggests that some structural rearrangement of connective tissue may take place in the cervical stroma of cows around oestrus.

## Cervical softening during pregnancy and parturition

Before the cervix can dilate, extensive changes have to take place in the connective tissue before the onset of parturition. This process, during which the cervix becomes soft on palpation, is called cervical ripening or maturation. When parturition begins, further softening progresses in a relatively short time, enabling the cervix to open under the force of uterine contractions. Changes in the structural arrangement of the collagen network of the cervical connective tissue during pregnancy (Junquiera *et al*., 1980; Yoshida and Manabe, 1990; Leppert, 1995; Parry and Ellwood, 1981) form the major causes of the loss of tensile strength of the cervix. Elastin is considered to be less important than collagen for cervical resistance against mechanical forces. In the human cervix elastin concentrations represent a minor fraction of the total connective tissue (Leppert and Yu, 1991).

The relative concentration of various glycosaminoglycans in the interstitial matrix may contribute to the regulation of the visco-elastic properties of the connective tissue. Decorin, a small proteoglycan which has a stabilising effect on the collagen network, is more abundantly present in non-pregnant and early pregnant cervices than in the cervix of term and parturient women (Winkler and Rath 1999). The concentration of hyaluronic acid, which has a high affinity for water, significantly increases in the cervix of women during labour (Rath *et al*., 1994) and disperses the collagen molecules by attracting more water to the interstitial space. Hyaluronic acid along with keratan sulphate may also stimulate the induction of cytokine production such as interleukin-1 (IL-1) (Mori and Ito, 1991). IL-1, IL-8 and other cytokines are known to induce a cascade of degradative processes in the connective tissue, through attraction and activation of leucocytes and lymphocytes and by stimulating the release of proteolytic enzymes and toxic metabolites from these leucocytes (Chwalisz *et al*., 1994; Sennström *et al*., 1997; Belayet *et al*., 1999; Ramos *et al*., 2000). In the cow, maternal arterial plasma levels of IL-1 were found to increase some 14 h after PG injection to induce parturition (Koets *et al*., 1998). Nitric oxide (NO) has been shown to increase dramatically during the active stage of parturition in the rat cervix (Buhimschi *et al*., 1996). In addition to its involvement in the modulation of prostaglandins and mast cell function, NO has also been shown to cause cell death either by necrosis or apoptosis when present in the tissue in high concentrations (Buhimschi *et al*., 1996). Reports of increased stromal cell apoptosis during pregnancy and parturition in the human (Alaire *et al*., 2001) and rat cervix (Leppert and Yu, 1995) suggest that cervical cell apoptosis may be either a cause or an effect of cervical remodeling during the two stages.

In women, the degree of cervical dilatation appeared to correlate with the extent of neutrophilic infiltration and the concentration of collagenolytic enzymes such as metalloproteinases (collagenases) (Winkler *et al*., 1999). Metalloproteinases can be produced either by neutrophils, macrophages or fibroblasts (Cawston, 1996). Thus, depending on which cells are stimulated, the origin of the metalloproteinases may depend on the stage of pregnancy or parturition. Many factors that are involved in cervical softening are associated with inflammatory processes, which are potentially damaging to the tissue. The increased synthesis of tissue inhibitors of metalloproteinases (TIMPs) which occurs at almost the same time may prevent the tissue from becoming too severely damaged (Ito *et al*., 1991; Imada *et al*., 1994). TIMPs may also play a role in regeneration processes within the cervix during the postpartum period.

The balance between degradative/ catabolic and regenerative/ metabolic processes in the cervix is carefully orchestrated by several hormones, among which oestrogens, progesterone, prostaglandins and relaxin. Progesterone and oestrogen are thought to be important regulators of the different processes that lead to the structural changes in the cervical connective tissue. In general, progesterone is associated with processes that keep the connective tissue intact and prevent cervical softening. Blocking progesterone functioning by treatment with

progesterone antagonists, promotes cervical softening in women (Stjernholm *et al*., 1999) and several other species such as rats (Rechberger *et al*., 1996). Infusion of dexamethasone into the foetus of late pregnant cows that were simultaneously treated with progesterone failed to induce parturition, while the cervix remained firmly closed to open only after progesterone infusions were discontinued (Fairclough *et al*., 1984). Progesterone also up-regulates the production of TIMPs as shown in rabbits (Imada *et al*., 1994). Oestrogen is usually found to stimulate processes that are associated with cervical softening. Oestrogen stimulates the production of $PGE_2$ in the cervices of cows (Fuchs *et al*., 1996) and has also been shown to stimulate the infiltration of eosinophils in the rat cervix. However, this occurs only in the absence of progesterone (Luque *et al*., 1996). Oestrogen was also found to stimulate the activity of pro-collagenases in cervical cell cultures of guinea pigs, an action that was completely blocked by progesterone (Rajabi *et al*., 1990; Rajabi *et al*., 1991). These data support the clinical observation in most species, that the cervix is more rigid during the progesterone dominated phase of the cycle and the major part of gestation, than during oestrous and parturition.

Mechanical stress on the cervix caused by increasing uterine contractions was initially thought to play an important regulatory role on the final cervical softening, during parturition (Owiny, 1992). Reports that cervical softening in parturient sheep occurs in the absence of increased intra uterine pressure, when the cervix is surgically separated from the uterus (Ledger *et al*., 1985), or when uterine contractions are blocked with $\beta$-sympatico mimetics (Owiny, 1992), suggest that the cellular and biochemical processes that cause cervical softening are independent of uterine contractions. Uterine contractions, however, are necessary for the softened cervix to fully dilate.

## Pregnancy and parturition in cows

The mean gestational length in cows is about 280 days. The main source of progesterone during pregnancy is the corpus luteum. During the last weeks of gestation, plasma concentrations of unconjugated oestrogens in the mother increase, while progesterone concentrations gradually decline (Pope *et al*., 1969, Stabenfeldt *et al*., 1970; Edqvist 1973). When parturition is due, endogenous cortisol of adrenal origin stimulates the uterus to produce prostaglandins. When these prostaglandins reach the ovary, the corpus luteum regresses and the progesterone production suddenly diminishes. This takes place between 24 and 36 h before calving and prepartum luteolysis and is a prerequisite for normal calving (Taverne, 2001). The hormonal changes that take place during spontaneous parturition are very closely mimicked when in pregnant cows parturition at term is induced by means of $PGF_{2\alpha}$. The period of prepartum luteolysis has been associated with temporary uterine quiescence, the orogin of which is not well understood, but during which relaxin or a relaxin–like factor may play a role in the inhibition of myometrial activity and possibly also in softening of the cervix (Janszen *et al*., 1990). A relaxin–like factor gene has been isolated in the bovine

ovary (Bathgate *et al*., 1996) and a significant increase in maternal relaxin-immuno reactivity has been found around parturition (Anderson et al., 1995). Purified (porcine) relaxin has been described to induce cervical dilatation in late pregnant cows, without inducing parturition (Perezgrovas and Anderson, 1982). These observations suggests the presence of relaxin receptors in the cervix. Such receptors have not been demonstrated in the bovine cervix. During the period of temporary myometrial quiescence, maternal plasma PGFM concentration already begin to increase (Janszen *et al*., 1990; Koets *et al*., 1996), which may not only reflect the increase of uterine $PGF_{2\alpha}$ production, but also of the cervical $PGE_2$ production.

## Objectives of the thesis

In this thesis, functional aspects of the cervix of the cow were studied during the non-pregnant, the pregnant and parturient state, using two different methodological approaches. A cellular and biochemical approach was followed in chapters 2, 3 and 4, while the studies described in chapter 5 and 6 used in-vivo monitoring of cervical dilatation, uterine electromyographic (EMG) activity and hormonal changes in maternal plasma.

Studies on cellular and biochemical changes in the cervical connective tissue are often difficult to compare with each other. Differences in sampling procedures and differences in the regions that are used for sampling, as well as the choice of control groups, which are not uniform in composition are responsible for this. In some of the studies in women, cervical samples have been obtained from the vaginal part of the cervix. In other studies they were obtained from the uterine side, during caesarean sections or hysterectomies. In animal studies, usually the entire cervix is removed. In the many reports that were studied for this thesis, only limited information about the depth of sampling or of which tissue parts of the samples were used, was provided. The marked differences in distribution of the tissue components along the cross-sectional as well as the longitudinal axis of the cervix and the differences in texture between oestrous and non-oestrous animals, prompt for a detailed description of sampling procedures and the endocrinological status of the control and study groups. In **chapter two** we adressed the question to what extent differences in texture of the cervices between non-pregnant cows with or without a functional corpus luteum or between different regions within the cervix, could be explained by differences in biochemical characteristics of the collagen in the stromal layer. To address this question, different sites along the cross-sectional and longitudinal axis of the cervix were sampled.

Oestrogen and progesterone receptors are present in the cervix. The biological effect of oestrogens and progesterone on the cervix depends not only on the plasma concentrations, but also on the concentration of their receptors, receptor genes and the ratio between different receptor isotypes in the tissue (Spencer and Bazer, 1995, Wang *et al*., 2001). In **chapter three**

immunohistochemistry was used to evaluate the localisation and distribution of the progesterone receptor and oestrogen receptor-$\alpha$, by studying different regions along the cross-sectional and longitudinal axis of the cervix in non-pregnant cows with and without a functional corpus luteum.

Cervical ripening and softening in several species have been reported to be associated with increased extractability of collagen from the cervical tissue and/ or with decreased collagen concentrations (human: Uldjberg *et al*., 1983; Stjernholm *et al*., 1996; sheep: Fitzpatrick 1977; cows: Kaidi *et al*., 1995). These reports were based on cross-sectional studies, which are sensitive to selection biass. The human studies often depend on biopsies that are obtained during gynaecological or obstetrical interventions, with non-uniform sampling. To overcome these problems, a longitudinal approach was followed in **chapter four**, where we repeatedly took biopsies of the caudal cervix during pregnancy and at spontaneous calving in the same cows. We always obtained the samples from the same region, approximately 2 cm cranial from the vaginal opening, from the luminal side of the cervix. Water content, collagen content, collagen concentration and percentage of collagen denaturation were measured in the stromal layer of the cervical tissues, of which the superficial part was always seperated from the deeper part.

There is still a lack of information on the temporal relationship between peripheral hormonal changes, onset of myometrial activity, onset of cervical dilatation and dilatation rate. More insight in these relationships would greatly improve our ability to studie the cellular and biochemical changes during cervical ripening and dilatation, because it would allow us to relate them more accurately to the beginning and/ or specific stage of the parturition process. Therefore, in **chapter five and six**, cervical dilatation was studied in vivo using chronically instrumented cows in which parturition was induced by injecting a synthetic $PGF_{2\alpha}$ analogue. Chapter five reports on the adaptation of an ultrasonic cervimetry technique for the use in cows, that was originally developed to study cervical dilatation in women. In chapter 6 we combined the same technique with in vivo EMG recordings of the myometrium and with analysis of hormonal changes in the maternal plasma after $PGF_{2\alpha}$ induced calvings, to assess the temporal relationship between cervical dilatation, uterine activity and hormone levels. In the summarising discussion (**chapter seven**), we discuss the implications of the results found in the chapters 2, 3 and 4. We also discuss possibilities to use the experimental model from chapters 5 and 6, for future studies to address some of the questions that were raised as a result of the first three studies.

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**-chapter II-**

# Regional differences in water content, collagen content and collagen degradation in the cervix of non-pregnant cows

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-submitted for publication-

## Abstract

During the oestrous phase of the cycle, the bovine cervix becomes softer in texture than during the luteal phase. It was explored if changes in the collagen network take place that could be responsible for this phenomenon and also, if regional differences in water content, collagen content and collagen degradation along the cross-sectional and longitudinal axis of the cervix were present. Two groups of non-pregnant 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 lyophilisation. The collagen content, and ratio of collagenous to non-collagenous proteins (hydroxyproline/ proline ratio), was determined by performing amino-acid analysis on hydrolysed samples using HPLC. Collagen denaturation was quantified with a colorimetric assay by determining the amount of hydroxyproline released from samples treated with $\alpha$-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 segment but this was unrelated to the progesterone status of the animals. There was no effect of the tissue layers or of the progesterone status of the animals on the collagen content, but there was an effect of segment. The collagen content ($\mu$g/mg dry weight) in the vaginal segment of the cervix was significantly higher than in the mid ($p < 0.05$) and the uterine ($p < 0.01$) segment. 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 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 non-pregnant cows, which may account for the difference in firmness of different parts along the circular or the longitudinal axis of the cervix.

## Introduction

During the follicular phase of the cycle the cervix of the cow becomes markedly softer than during the luteal phase (Hafez, 1973). This is also the case in species such as the horse (Hafez, 1973) and the dog (Silva *et al*., 1995). By contrast, in the pig the cervix is stiff during oestrus and becomes softer during the luteal phase (Meredith, 1977). Also at parturition, the cervix has to soften before it can dilate. This process is known to be caused by biochemical events that lead to structural changes in the connective tissue of the cervix, especially in its collagen network (Hafez, 1993). 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 due to its cross-links (Bank *et al*., 1997) that 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 on the importance of connective tissue changes during softening of the pregnant cervix, seemingly contradictory mechanisms have been reported such as collagen loss (Rath *et al*., 1994) versus increased collagen synthesis, combined with increased synthesis of proteoglycans and hyaluronic acid (HA) (Leppert and Yu, 1991; Huang *et al*., 1993). Although many factors are involved, it is 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 oestrous 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 axis of the pregnant as well as the non pregnant cervix have also been described in different species (Harkness and Harkness, 1959; Hafez, 1973; Krantz, 1973; Ferenczy, 1980; Koob and Ryan, 1980; Koob *et* al., 1980; Leppert, 1995; Fuchs *et al*., 1996). Due to the morphological differences between the layers, functional differences in speed and extend of softening in different parts of the cervix might be expected. This fact has been clearly demonstrated in the pregnant sheep by the observation that only the dense collagenous connective tissue changes into a soft gel when the cervix ripens (Owiny *et al*., 1992). 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 also that the uterus itself has no influence on this process (Ledger *et al*., 1985). Similarly, in pregnant pigs, biochemical and biomechanical studies revealed that softening does not proceed at the same degree and with the same speed at different sites along the longitudinal axis of the cervix (O'Day *et al*., 1989; O'Day-Bowman *et al*., 1991; Winn *et al*., 1993).

Few and contradictory data on the distribution of and about changes in the collagen network are available on the non-pregnant cervix (Conrad *et al*., 1980;

Conrad and Hoover, 1982; Petersen *et al*., 1991; Scout *et al*., 1993; Winn *et al*., 1994). Petersen *et al*. (1991) did not find any significant differences in hydroxyproline concentration between the vaginal and uterine end of the human cervix. Neither did they find any differences in passive bio-mechanical properties between the vaginal and uterine end nor between preparations which were cut parallel or perpendicular to the cervical canal. This is in contrast with the report of Conrad and Hoover (1982) on rabbits, who observed significant differences in passive bio-mechanical properties between the vaginal and uterine end. In addition, Conrad *et al*. (1980) found a significant decrease in stretch modulus from the lumen to the outer wall of the human cervix.

Collagen is a major component of the connective tissue. Therefore, the distribution, content and degree of degradation of collagen may have a regulatory influence on softening of the cervix. In the case of in vivo studies, where sampling is only possible from the caudal cervix, it is important to know if the tissue obtained is representative for the rest of the cervix. In the cow, such data are lacking. In this study we aimed to asses 1) if regional differences in collagen biochemistry are present along the cross-sectional or longitudinal axis of the bovine cervix and 2) if significant differences exist in the collagen network between animals under high or low progesterone influence, that may possibly explain the softening of the bovine cervix during the low progesterone phase of the oestrous cycle.

## Materials and Methods

*Reagents*

Guanidium chloride (GuHCl), EDTA, iodoacetamide, $\alpha$-Chymotripsine ($\alpha$CT), 9-fluorenylmethyl chloroformate (FMOC), pyridoxine mono-hydrochloride, homo-arginine and the amino acid standard for collagen hydrolysates were obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid 37% (or 12M), sodium acetate trihydrate, sodium hydroxide p.a., acidic acid, 2-propanol, chloramine-T, dimethylaminobenzoaldehyde (DMBA), perchloric acid 60% and hydroxyproline were purchased from Merck (Darmstadt, Germany). Heptafluorobytiric 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 was 10 $\mu$M and 2.4 mM, respectively. The amino acid standard was diluted together with homo-arginine in 0.1M borate buffer pH 8.0, so that the injected volume in the HPLC contained 250 pmol of proline and hydroxyproline, 25 pmol cystine and 100 pmol homo-arginine.

Incubation buffer consisted of 1mM iodoacetamide and 1mM EDTA in PBS, pH 7.5. A solvent of 4M GuHCl in incubation buffer was used for extraction of proteoglycans and soluble collagen. Digestion buffer was made by dissolving 1mg/ ml $\alpha$CT in incubation buffer. Stock buffer pH 6.1, contained 50.44 g/ l citric acid,

117.76 g/ l sodium acetate trihydrate and 34 g/ l sodium hydroxide p.a. Assay buffer was made by mixing stock buffer with 2-propanol and de-ionized water in a ratio 10:3:2 (v:v:v). Chloramine-T reagens contained 0.141g chloramine-T dissolved in 1ml 2-propanol, 1 ml de-ionized water and 8 ml stock buffer. DMBA reagens contained 4 g DMBA in 2.5 ml 2-propanol and 5.5 ml 60% perchloric acid. The 200 μM hydroxyproline standard contained 26.23 μg/ ml 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 their respective sizes, the specimens were divided in a non-luteal group and a luteal group. Selection of specimens for the non-luteal 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 and firm cervix and no mucus present in the vagina. Afterwards, a finer selection was made into a high progesterone and a low progesterone group, based on serum progesterone levels and these groups were further used in this study. Only luteal cervical samples from animals with a serum progesterone > 2ng/ml, were finally accepted into the high progesterone group. Only non-luteal samples from cows with a serum progesterone ≤ 0.5ng/ml, were finally accepted into the low progesterone group. The progesterone concentrations were measured by a validated direct solid-phase $^{125}$I RIA method (Dieleman and Bevers, 1987). Sensitivity of the assay was 47 pg/ ml; the inter-assay coefficient of variation was 11% (n = 16) and the intra-assay coefficient of variation was 7.5% (n = 20).

Circular slices of approximately 5 mm thickness were cut from the vaginal (V), middle (M) and uterine (U) 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 uterine mucosa. The vaginal segment was obtained approximately 1 cm cranial from the external os of the cervix, and the mid-segment was obtained halfway the two former sites. Samples were taken from the superficial stromal layer (S) 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 (D) consisting of the more peripheral fibrous tissue (see fig 1B). Samples were frozen in liquid nitrogen and kept at - 80 °C, until further processing.

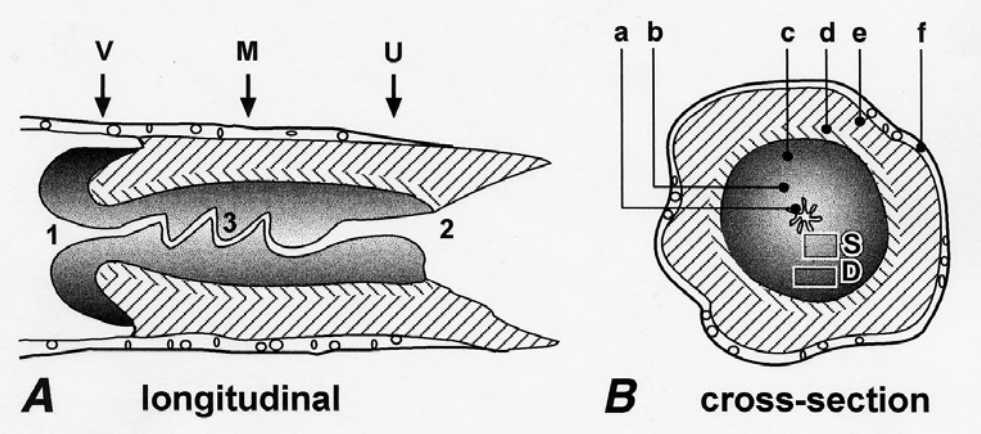


Fig. 1. Schematical pictures of the bovine cervix. **A**) longitudinal section of the cervix: V = vaginal, M = mid, and U = uterine segment, 1 = portio vaginalis (external os), 2 = uterine lumen, 3 = cervical canal. **B**) cross-section of the cervix: Small samples from the superficial (S) and deep (D)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

*Isolation of collagen from cervical tissue and morphology*

Tissues from the cervical mucosa of three animals (two luteal and one non-luteal) were incubated overnight at 4 °C with 1 mg/ml pepsin in 0.5 M acetic acid (tissue/enzyme ratio [wet wt/v], 1:25). The released collagen was subsequently loaded on 7.5 % sodium dodecyl sulphate (SDS) polyacrylamide gels, subjected to interrupted gel electrophoresis, and stained with coomassie brillant blue as described previously (Sykes *et al*., 1976). Collagen type I and III isolated from human placenta 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 segment of the cervix of four high progesterone and two low progesterone animals were placed in 4% buffered formaldehyde for 48 hours and embedded in paraffin. Sections of 6 μm were mounted on glass slides and stained with periodic acid schiff (PAS) and hematoxylin.

*Tissue preparation for biochemical analysis*

From each cervix, six different sites were analysed (vaginal superficial, vaginal deep, mid superficial, mid deep, uterine superficial, uterine deep). For all 23 different animals (high progesterone n = 11, low progesterone n = 12), the six sites were analysed except for a few cases indicated in the materials and methods section and specified under statistical analysis, were samples appeared 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 of denaturation of the collagen in duplicate, and the other two were used for amino-acid analysis in duplicate (analysis of collagen content, see below).

*Water content*

All tissue samples that were used for the determination of the percentage denaturation and for the amino-acid analysis were rinsed in isotonic saline, blotted dry, and weighed to obtain the wet weight. They were then lyophilised and weighed again to obtain the dry weight. After this, the water content could be calculated (wet weight minus dry weight) 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 the statistical analysis paragraph. The wet weight ranged from 50 mg to 200 mg, and the dry weight ranged from 6 mg to 37 mg. After lyophilisation the samples were further processed.

*Analysis of collagen content*

Collagen content was determined by measuring the amount of hydroxyproline present in the duplicate samples, according to the method of Bank *et al*. (1996,1999). The means of the duplicate samples were used for statistical analysis. Briefly, lyophilised samples were hydrolysed in 6M HCl for 24 h at 110°C and dried. The samples were dissolved in an internal standard solution containing 2.4 μmol homo-arginine per ml water and were diluted 5 fold with 0.5 % (v/v) HFBA in 10% (v/v) ACN. They were further diluted 50 fold with 0.1M borate buffer, pH 8.0. Then 200 μl samples were derivatised with 200 μl 6mM FMOC and extracted twice with 600 μl pentane. Following this, 400 μl 25 % (v/v) ACN in 0.1 M borate buffer pH 8.0 was 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 hydroxyproline/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, as non-collagenous proteins contribute to the released proline pool. As such, the hydroxyproline/proline ratio reflects the ratio of collagenous to non-collagenous protein.

Because, some of the hydrolysates were lost during drying due to technical problems., it was not possible to analyse 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 earlier by Bank *et al*. (1997). Briefly, proteoglycans and soluble collagen were removed from the

tissue by extracting tissue samples twice for 24h with 4M guanidinium chloride (GuHCl) in incubation buffer at 4°C. After washing in incubation buffer for 3 times to remove GuHCl, the denatured collagen in the insoluble tissue matrix was digested overnight at 37°C in 1 ml digestion buffer. The supernatant containing the αCT-solubilized collagen fragments was removed quantitatively and diluted 1:1 with 12M HCl. The remaining tissue was immersed in 800 µl 6M HCl. Supernatant and residual tissue were hydrolysed at 110°C for 24h and dried. After drying, the hydrolysates were dissolved in 1 ml water. The hydroxyproline (Hyp) 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 (1967) and as described by Creemers *et al*. (1997). In short, the hydrolisates of the supernatant and of the remaining tissue were diluted 10 and 50 times respectively. From these samples, 60 µl was pipetted into a well of a polystyrene microtiter plate, after which 20 µl assay-buffer and 40 µl chloramine-T reagent were added. After a 20 min incubation at room temperature, 80 µl DMBA reagent was 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 minutes. After this, the plate was cooled down by placing it in a water bath containing cold water for 5 min, which was refreshed once during that time, and the extinction was measured at 570 nm on a Titertek multiscan MCC/340 (Labsystems, Finland). A hydroxyproline standard series (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 µM) and blancs (water) were included in the measurements. The extinction of the standards and the samples was first corrected for the extinction of the blancs. If the extinction of samples were 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 with the following formula:

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

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 analysis in Table 1. All data were analysed with the GLM procedure of SAS (1990), using the following model:

$$Y_{ijk} = \mu + P4_i + e1_{ij} + Segm_j + Depth_k + e2_{ijk}$$

This model tested the differences between the high progesterone group (HP4) and the low progesterone group (LP4) against the variation between animals and the differences between segment and depth of the cervix against the variation within animals, where $Y_{ijk}$ = dependent variable; $\mu$ = overall mean; P4 = progesterone level (i = high, low); $e1_{ij}$ = error term 1, which represented the random effect of animal j ( j = 1 to 23), nested within progesterone level; $Segm_j$ = segment of cervix (j = vaginal, mid, uterine); $Depth_k$ = cross sectional depth (k = superficial, deep) and $e2_{ijk}$ = residual error. Firstly, all interactions were tested for significance. Non significant interactions were excluded from the model, after which significant interactions were re-evaluated. All data presented are means $\pm$ sem. Significance was accepted at the $p < 0.05$ level.

Table 1. Number of animals used for each site of the cervix, specified for each group and for the different types of biochemical analysis that were performed on the non-pregnant bovine cervix.

| | | Vaginal superficial | Vaginal deep | Mid superficial | Mid deep | Uterine superficial | Uterine deep |
|---|---|---|---|---|---|---|---|
| Water content | HP4 | 11 | 11 | 11 | 11 | 10 | 10 |
| | LP4 | 12 | 12 | 12 | 12 | 12 | 12 |
| Collagencontent hydroxyproline/proline | HP4 | 7 | 7 | 7 | 7 | 5 | 5 |
| | LP4 | 12 | 12 | 10 | 10 | 10 | 10 |
| Percentagecollagen denaturation | HP4 | 11 | 11 | 11 | 11 | 9 | 10 |
| | LP4 | 12 | 12 | 12 | 12 | 11 | 11 |

HP4 = high progesterone group
LP4 = low progesterone group

## Results

*Typing of collagen, microscopic and gross morphology*

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

The cervices of the low progesterone group felt softer by palpation and appeared more hydrated than those of the high progesterone group. Macroscopically it was observed that at the vaginal side, the muscle layer was relatively thinner than at the uterine side, while 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 on 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).

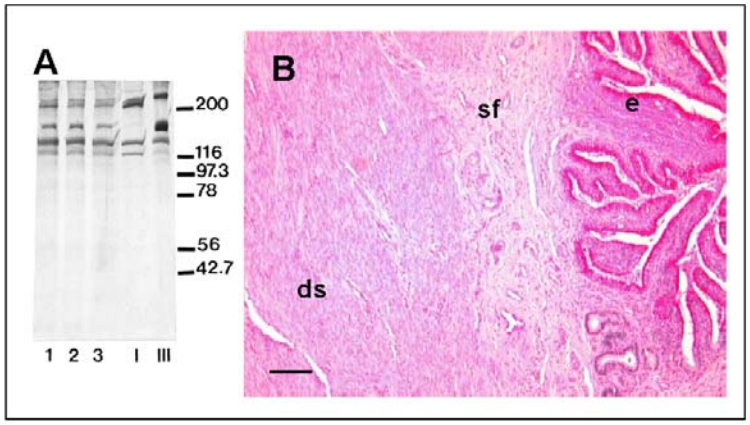


Fig. 2. **A**) Collagen type in the cervix of three cows was analysed by SDS-PAGE: 1, 2 and 3 are the samples from three different cows. I and III are the controls for collagen type I and III. **B**) Photomicrograph from the mucosal area of the cervix. PAS/ hematoxilin staining, showing the deep stromal layer (**ds**), superficial loose stromal band (**sf**) and columnar epithelium (**e**). Scale bar represents 14 μm.

*Water content*

There was a significant relationship 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, there was no effect of the progesterone status of the animals or of the segment along the longitudinal axis of the cervix on the water content.

*Collagen content and ratio of collagenous to non-collagenous proteins (hydroxyproline/proline)*

There was a significant effect of segment ($p < 0.0001$), but not of the depth of the tissue layer or the progesterone status on the collagen content. Because there were no differences between the high progesterone and low progesterone groups or between tissue layers, the data where pooled to analyse the effect of the segment. The vaginal segment had a significantly higher collagen content than the mid ($p < 0.05$) and uterine segments ($p < 0.01$, Fig. 4A).

Similarly, no relation was found between the tissue layers and the progesterone status on the hydroxyproline/ proline ratio, but there was an effect of the segment ($p < 0.0001$). Therefore, the data were also pooled to analyse the effect of segment. At the vaginal segment there was a significantly higher hydroxyproline/ proline ratio than at the mid ($p < 0.05$) and the uterine segment ($p < 0.01$, Fig. 4B).

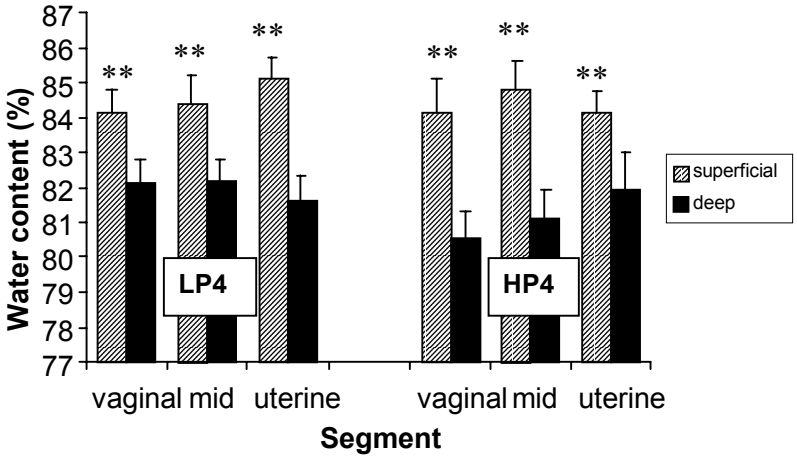


Fig. 3. Water content (% of wet weight, mean ± sem) in three different segments along the longitudinal axis of the bovine cervix. LP4 = low progesterone group, HP4 = high progesterone group. Asterisks indicate a significant difference in water content between the superficial and deep stromal layer of each segment (** $p < 0.01$).

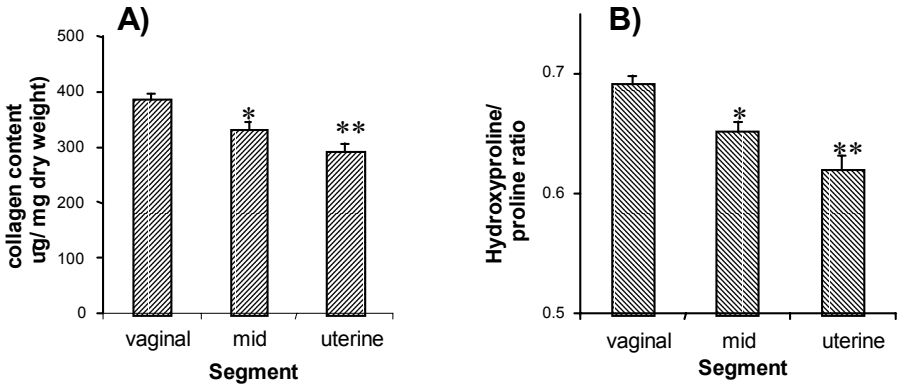


Fig. 4 **A**) The collagen content (mean ± sem) and **B**) the ratio of collagenous to non-collagenous protein (hydroxyproline/ proline ratio, mean ± sem) of pooled values of the superficial and deep layers from both high progesterone and low progesterone group, in three different segments of the bovine cervix. Significant differences compared to the vaginal segment are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

*Percentage of denaturation*

There was a significant relationship 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).

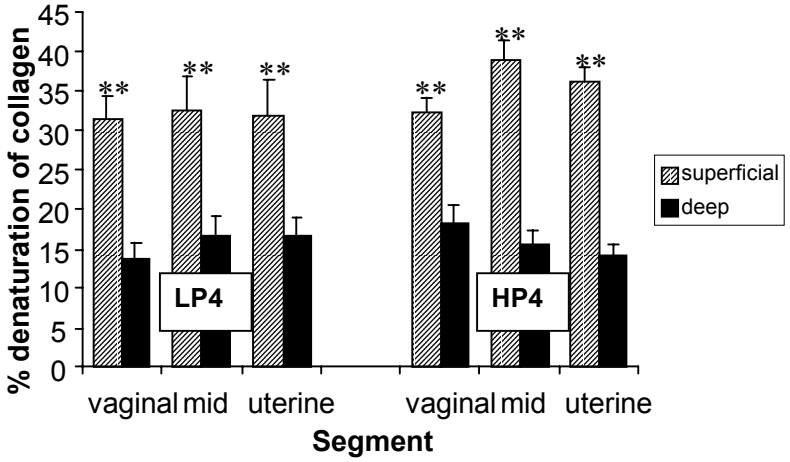


Fig. 5. The percentage of denaturation of the collagen (mean ± sem) in three different segments along the longitudinal axis of the bovine cervix. LP4 = low progesterone group, HP4 = high progesterone group. Within the same segment asterisks indicate a significant difference in percentage denaturation between the superficial and deep stromal layer (** $p < 0.01$).

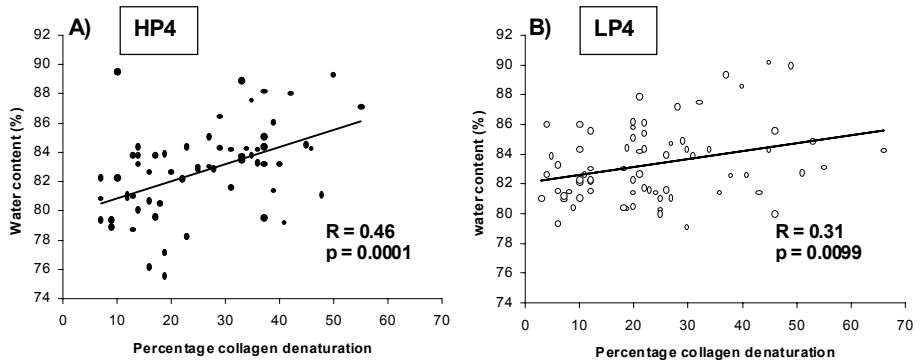


Fig. 6. Correlation between the percentage of denatured collagen and the water content of the cervix. **A**) in the high progesterone (HP4) group and **B**) the low progesterone (LP4) group

Also, a positive linear relationship was found between the water content and the percentage denaturation (R = 0.36, p = 0.0001). The correlation was stronger in the high progesterone group (R = 0.46, p = 0.0001, Fig 6A) than in the low progesterone group (R = 0.31, p = 0.0099, Fig 6B).

## Discussion

This study demonstrates 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 non-pregnant cows in both the presence and the absence of a functional corpus luteum. Moreover, differences in collagen content and the ratio of collagenous to non collagenous proteins were found between the vaginal and uterine segment of the cervix. Our finding that the bovine cervix contains both collagen type I and III, is in agreement with the observation in the human cervix (Kleisl *et al*., 1978).

A decreasing collagen content was found from the vaginal towards the uterine side of the cervix in both the low progesterone and the high progesterone group, whereas, along the cross-sectional axis there were no differences in collagen content between the superficial and deep layer. The subjectively observed difference in softness between the two layers during collection of the cervix samples, can not 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 (Petersen *et al*., 1991), but not with the reported fluctuations in total collagen content of the cervix during the oestrous cycle of the mouse (Rimmer, 1972).

There are major differences in collagen content of the cervix between species. When the collagen content as found in the present study is expressed as a percentage of dry weight, it measures about 38% at the vaginal side and about 29% at the uterine side. These figures are higher than those reported for the mouse (16%) (Rimmer, 1972), within the same range as in sheep (44%) (Fitzpatrick, 1977) and rabbits (42%) (Koob *et al*., 1980), but they are much lower than in rats (60 - 70%) (Knudsen *et al*., 1998), or humans (50 – 85%) (Danfort and Buckingham, 1973; Uldjberg *et al*., 1983; Leppert and Yu, 1991). In contrast to our results, the human cervix appears to have a higher collagen content at the uterine end than the vaginal end (Leppert and Yu, 1991), although yet another report claimed that there are no differences in collagen content (Petersen *et al*., 1991) along the longitudinal axis of the non pregnant human cervix. However, expressing the collagen content relative to dry weight as Leppert and Yu (1991) did, may give different results as when expressed relative to wet weight as did Petersen *et al*. (1991).

Our findings indicate that the soft appearance of the superficial stromal layer is 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. Structural changes do not correlate well with changes in collagen content or concentration, as was demonstrated by Junqueira *et al*. (1980) who observed, by means of histochemical analysis, that the loss of structure in the collagen network (fibre length and orientation) in parturient women was much more pronounced than the amount of collagen would suggest. The observation of Conrad *et al*. (1980) that different zones along the circular axis of the non-pregnant human cervix had the same collagen content, whereas the stretch modulus decreases significantly from the lumen towards the outer wall, also support our findings. Reported differences in collagen extractability after pepsin-digestion in the softened human cervix (Granström *et al*., 1989; Granström *et al*., 1991; Stjernholm *et al*., 1996; Petersen and Uldjberg 1996; Stjernholm *et al*., 1997) also suggest that degradation of the collagen network alters its structure so that it can be more easily extracted.

Exogeneous oxytocin is known to cause softening of the non-pregnant cervix in oestrous sheep (Khalifa *et al*., 1992), most probably through facilitation of local $PGE_2$ production (Norström *et al*., 1981; Duchens *et al*., 1993; Fuchs *et al*., 1995; El Maradny *et al*., 1996). Regional and cyclical differences in expression of the oxytocin receptor in the bovine (Fuchs *et al.,* 1996) and ovine cervix (Matthews and Ayad 1994) have been reported. According to Fuchs *et al*. (1996), the oxytocin receptors in the bovine cervix are mainly situated in the mucosal layer, and especially the epithelial receptors are significantly upregulated during the oestrous phase. If these findings are extrapolated to our results, one may speculate that, especially in the superficial layer, locally produced $PGE_2$ acting in a paracrine way causes collagen degradation in the tissue directly underlying the epithelium, thereby explaining the higher percentage denaturation in the superficial layer. On the other hand, our study does not provide any evidence for a higher percentage denaturation within the superficial layer in animals of the low progesterone group than those of the high progesterone group. So, the existence of a functional relationship between up-regulation of oxytocine receptors within the luminal surface epithelium and the connective tissue biochemistry of the underlying stroma needs further investigation.

A significant increase in the water content of the porcine cervix occurs shortly after a two days exposure to oestrogens, and similar results were found in pigs which had been given oestrogen daily over a more prolonged period (Hall and Anthony, 1993). These observations suggest that an increase in circulating oestrogens stimulates the attraction of water into the cervical tissue. Other authors (Huang *et al*., 1997), however, found no effect of oestrogen on the water content of the porcine cervix, when oestrogen, without progesterone was administered every other day during a 2-week period. On the basis of ovarian morphology it can be assumed that the cows in our low progesterone group have been slaughtered during or shortly after a period of oestrogen dominance and that the stimulatory effect of the oestrogens on the cervical water content might still be present. Nevertheless, our results did not show significant differences in water content

between the low progesterone and high progesterone group. On the other hand, the observation that the superficial layer clearly felt softer by palpation than the deep layer, and also had a significantly higher water content, indicates that the water content has an important influence on the texture of the cervix.

Apparently, the observed differences in water content between the superficial and the deep stromal layers of the cervix in both the high progesterone and low progesterone group are associated with the differences in collagen denaturation, although, we did not find an equally high correlation as was reported by Bank *et al*. (1996, 2000), who found that the swelling properties of cartilage are highly correlated with the amount of denatured (degraded) collagen. However, one should realise that the concentration or composition of other connective tissue constituents present within the cervix, other than collagen, can influence the texture. The differences between hydroxyproline/proline ratio of the cervical segments showed the same pattern as the collagen content (see Fig. 4A and B). In human cartilage the hydroxyproline/proline ratio is negatively correlated with the total glycosaminoglycan concentration in the tissue (RA Bank, personal communication). If the same applies for the cervix, this would implicate that the decreasing hydroxyproline/ proline ratio from the vaginal to the uterine segment is indicative of a similarly oriented increase in glycosaminoglycan content, which might cause differences in the texture of the cervical tissue (Andersen *et al*., 1991; Rath *et al*., 1994). As judged by their similar collagen content and hydroxyproline/ proline ratio, the total glycosaminoglycan content of the deep stromal layer is probably not different from the superficial stromal layer. However, it can be hypothesised that the composition of the glycosaminoglycan content in the superficial layers is more in favour of glycosaminoglycans with a higher water binding capacity as opposed 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 (Andersen *et al*., 1991). In line with this hypothesis, the ratio of glycosaminoglycans with higher water binding capacity to those with less water binding capacity could be higher in animals around oestrus. Similarly, decorin, which stabilises the collagen network (Winkler and Rath, 1999) may be present in lower concentrations in oestrous animals or may play a role in the difference in texture between the superficial layer and the deep stromal layer. This remains to be investigated for the cow.

In this 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 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 softening of the cervix. The results also showed significant differences in collagen content along the longitudinal axis of the cervix. These last 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.

## Acknowledgements

The authors are especially indebted to N.M. Soede from Wageningen University and J. van den Broek of the Faculty of Veterinary Medicine, for assisting them with the statistical analysis and to W. Bes, for the drawings of figure 1.

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**-Chapter III-**

# Immunohistochemical distribution of oestrogen and progesterone receptors and tissue concentrations of oestrogens in the cervix of non-pregnant cows.

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-submitted for publication

## Abstract

An immunohistochemical study of the expression of the oestrogen receptor-$\alpha$ (ER$\alpha$) and the progesterone receptor (PR) in different regions along the longitudinal and vertical axes of the cervix of non-pregnant cows was performed. Animals were separated into two groups depending on the presence or absence of a functional corpus luteum in their ovaries, as confirmed by plasma progesterone concentrations. Animals of the high progesterone group (HP4) had serum progesterone concentrations > 2.0 ng/ml (n = 6) and those of the low progesterone group (LP4) had serum progesterone concentrations $\leq$ 0.5 ng/ml (n = 4).

Significantly higher concentrations of oestrogen were found in the cervical tissue of animals in the LP4 group than those in the HP4 group (473 $\pm$ 53 vs.149 $\pm$ 46 pg/g wet weight: $p < 0.01$). Furthermore, there was a significant effect of tissue layer (epithelium to deep stroma) on the number of ER$\alpha$ ($p < 0.01$) and PR ($p < 0.05$) immunoreactive nuclei per 1000 cells. The proportion of cells expressing both receptor types increased from epithelium to subepithelial stroma ($p < 0.01$) and from subepithelium to deep stroma (ER$\alpha$, $p < 0.05$; PR, $p = 0.061$).

When the number of receptor positive cells was expressed per $mm^2$ tissue, differences between the subepithelial stroma and the deep stroma became even more marked. In addition, the vaginal part of the cervix had significantly more ($p < 0.01$) ER$\alpha$ and PR immunoreactive nuclei per 1000 cells than the uterine part, but these differences were no longer apparent when a correction was made for cell-density. A strong relationship between both longitudinal and cross-sectional positioning of tissue in the cervix and expression of both receptor types was shown, but ER$\alpha$ and PR immunoreactivity was independent of circulating progesterone, or local tissue oestrogen concentrations in the cervix. In addition, a strong correlation between ER$\alpha$ and PR expression in the subepithelial stroma ($R = 0.85$, $p < 0.01$) and the deep stroma ($R = 0.83$, $p < 0.01$) was evident. These results demonstrate that in studies of steroid hormone receptor expression in the cervix uteri, careful description of sampling site and depth are necessary if the results are to be interpreted meaningfully.

## Introduction

The presence of oestrogen and progesterone receptors (ER and PR) in the uterine cervix, and the characteristic changes that this organ undergoes during the oestrous cycle and pregnancy, have identified it as a target for both of the important reproductive steroid hormones. Moreover, the expression of ER and PR in the cervix has been reported to vary according to the stage of the oestrous cycle in sheep (Zhao *et al*., 1999) dogs (Vermeirsch *et al*., 1999; Vermeirsch *et al*., 2000) and rats (Wang *et al*., 2000). Similarly, in non-pregnant cows (Vesanen *et al*., 1991; Vesanen 1993) and horses (Re *et al*., 1995), cervical cytosolic PR, but not ER, concentrations have been reported to be suppressed by high serum progesterone levels. In contrast, there appears to be no clear relationship between immunohistochemically detected ER or PR and the stage of the menstrual cycle in women (Kuprya□czyk and Möller, 1988; Cano *et al*., 1990; Kuprya□czyk, 1991; Snijders *et al*., 1992).

It has been reported that expression of both ER and PR in the cervix differs in different tissue layers and cell types (Kuprya□czyk and Möller, 1988; Cano *et al*., 1990; Snijders *et al*., 1992; Zhao *et al*., 1999) and in different regions along the longitudinal axis (Kuprya□czyk and Möller 1988; Cano *et al*., 1990), although these reported differences were based on semi-quantitative or qualitative descriptions. Interestingly, regional differences in the expression of the oxytocin receptor within the bovine cervix also vary with the changing steroid environment (Fuchs *et al*., 1996). Our recent observations that the mucosa of the bovine cervix is divided into two stromal layers that are clearly different in collagen biochemistry (chapter 2) and cell composition (Breeveld-Dwarkasing *et al*., 2000), suggest that regional differences in the expression of steroid hormone receptors are likely. These findings, together with the reports of cyclical and regional differences in receptor expression in other species, led us to investigate whether there is an association between serum progesterone concentrations and the expression of ER and PR in different regions of the bovine cervix. Immunohistochemistry was used to assess receptor expression in the epithelium, the subepithelial and deep stromal layers of the vaginal, middle and uterine segments of the cervix, in cows with either high or low serum progesterone concentrations. Local tissue oestrogen levels were also determined in the different sections of cervical tissue to determine to what extent they varied with ER$\alpha$ or PR expression.

## Materials and methods

*Sample collection*

Cervices were recovered from non-pregnant Holstein Friesian cows after slaughter. Immediately after stunning, jugular vein blood samples were collected from each cow for determination of the plasma progesterone concentrations by means of a validated, direct solid-phase $^{125}$I RIA (Dieleman and Bevers, 1987)

sensitivity of the assay was 47 pg/ml, the inter-assay coefficient of variation (CV) was 11% (n = 16) and the intra-assay CV was 7.5% (n = 20). Based on their plasma progesterone (P4) concentrations, the cows were divided into two groups: a low progesterone group (LP4) with plasma P4 concentrations $\leq$ 0.5 ng/ml (n = 4) and a high progesterone group (HP4) with plasma P4 concentrations > 2.0 ng/ml (n = 6). Care was taken to ensure that the progesterone concentrations correlated with other features of an oestrous stage such as ovarian morphology (presence, size and texture of the corpus luteum and follicles) and the macroscopic features of the cervix, such as abundant clear mucoid discharge. Circular slices were cut from the vaginal (V), middle (M) and uterine (U) segments of each cervix. From these slices, smaller wedge shaped pieces were cut, fixed in 4.5% formaldehyde for 48 hours and embedded in paraffin. Tissue samples were taken from each region, snap-frozen in liquid nitrogen and stored at - 80°C until assessed for their concentrations of oestrogens (see below).

*Local tissue oestrogen concentrations*

Initially, HPLC analysis was performed in a few samples, to identify the various oestrogen metabolites present in cervical tissue. The - 80°C frozen samples of the superficial layer (S = epithelium and subepithelial stroma) and the deeper stromal layer (D) from the three cervical segments (V, M, U) were used for the analysis of tissue oestrogen concentrations. Tissue extracts were made by mincing 0.2 g of the tissue in an eppendorf cup, adding 100 µl water and heating the mixture at 95 °C for 10 min to destroy steroid converting enzymes. Next, 1 ml of Hanks buffer containing 4mg/ml collagenase, 1.6 mg/ml dispase and 0.24 mg/ml DNAse - filtered through Sep pak R C18 cartridges (Waters®, Millford, Massachusets, USA) to remove any interfering substances- was added and the samples were incubated for 22 hours at 37 °C. After incubation, the samples were centrifuged (5 min, 9000 g) and 1 ml of the supernatant was transferred into a fresh tube. The pellet was re-suspended in 100 µl methanol and then vortexed, centrifuged and re-suspended once more in 100 µl methanol, with the second supernatant having been added to that from the first centrifugation. Next, an additional 2 ml of water was added and the aqueous phase was re-extracted using Sep pak R C18 cartridges. After priming and extraction, the steroids were removed from the cartridge using 80 % methanol. To determine the total oestrogen concentration in the tissue extracts, a biotin-streptavidin EIA kit that employed a sheep anti-17$\beta$ oestradiol-17-HS antibody was used, as described by Palme and Moestl (1993). These authors reported cross reactivity for this antibody of 100% with oestrone, 70% with oestradiol 17-$\beta$, 19% with oestradiol 17-$\alpha$ and < 0.01% with oestradiol-sulfate. The sensitivity of the assay was 0.7 fmol/ well and the inter- and intra-assay CVs, were 14.3% (n = 50) and 12.4% (n = 20), respectively.

*Immunolocalisation of ER and PR*

The mouse anti-human ER$\alpha$ antibody (clone ID5) and the mouse anti-human PR antibody (clone 10A9) were both obtained from BioGenex® (San

Ramon, USA). The anti ERα was a ready to use solution and the PR was diluted 1:50 with BSA-C/TBS (50 µl of a 10% acetylated bovine serum albumin, diluted in 10 ml TRIS buffered saline) before use. From wedges obtained at each cervical segment three 5 µm thin sections were cut, 100 µm apart, and mounted on a single, coated glass slide (Superfrost plus, Erie Sc. Co., Portsmouth, New Hampshire). Sections were deparaffinized with xylene and rehydrated with ethanol containing increasing concentrations of water, and finally deionized water. Endogenous peroxidase activity was quenched with 3% peroxide in deionized water. To expose the antigens, sections were microwaved for three 5 min periods at 780 Watts in a household microwave and, thereafter, 10% normal goat serum was used as a blocking antibody. The sections were incubated with the anti-ER or PR antibodies overnight at 4°C after which a biotinylated goat anti-mouse second antibody was applied to the sections, which were incubated for a further 1 hour. Next, the slides were incubated for 1 hour with streptavidin-biotin (ABC-elite kit) and finally, 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used to visualize the immunoreaction. The slides were counterstained with Mayers hematoxylin. For negative controls, sections of the cervix were incubated with normal mouse serum instead of a primary antibody.

*Proportional scores of ER and PR (number of positive nuclei per 1000 cells).*

The number of nuclei that stained positively for ER or PR in 1000 cells was counted at a magnification of 400× in three different layers along the cross-sectional axis. These three layers were 1) the surface epithelium, 2) the subepithelial stroma and 3) the deeper stroma. In each of the three tissue sections mounted on each glass slide, approximately 333 cells were counted to make a total of one thousand cells per layer. Any nucleus showing evidence of the DAB reaction product was considered to be positive, irrespective of the intensity of DAB staining. In this way, a proportional score of receptor positive nuclei (ERα or PR immunoreactive nuclei per 1000 cells) was obtained for the three different layers and the three different segments of the cervix each animal of both groups.

*Cell density and number of ER and PR positive nuclei per mm$^2$*

The cell density of the subepithelial stromal layer was compared to that of the deep stromal layer by counting all fibroblast and muscle cell nuclei in an area equivalent to 2 mm$^2$ of the tissue section, using a 1 cm$^2$ grid at 400× magnification. The mean number of cells per mm$^2$ was calculated from triplicate countings. These numbers were then used to estimate the number of ER and PR immunoreactive nuclei per mm$^2$ (ER per mm$^2$ and PR per mm$^2$) using the following formula:

$$\textit{Number of ER or PR immunoreactive nuclei per mm}^2 = (A / 1000 \times 100\%) \times B$$

Where A = number of immunoreactive nuclei per 1000 cells and B = number of cells per mm$^2$.

*Statistical analysis*

All the data are expressed as least square means (LSM) ± sem. The proportion of ER and PR positive nuclei in the epithelial, subepithelial stromal and deep stromal layers, and the number of ER and PR positive cells per $mm^2$ of the subepithelial stromal and deep stromal layers, were analysed using a General Linear Models procedure (SAS, 1990). The same model was used to examine whether there was a relationship between cell density and location within the cervix or between cell density and progesterone status. The differences between HP4 and LP4 were tested against the variation within animals, nested within progesterone level. Non-significant interactions were removed from the model while significant interactions were studied in more detail. In addition, the correlation between the number of ER$\alpha$ per $mm^2$ and PR per $mm^2$, and between receptor expression per $mm^2$ and the local oestrogen concentrations in the different sites were examined using Pearson's test. Differences were accepted to be statistically significant if $p < 0.05$ and were described as a tendency if $0.05 < p < 0.10$.

## Results

*Serum P4 concentrations and local tissue concentrations of oestrogens*

The serum P4 concentrations ranged from 2.1 - 5.7 ng/ml in the HP4 group and from 0.1 - 0.5 ng/ml in the LP4 group. HPLC analysis of cervical tissue demonstrated that oestrone and oestradiol 17-$\alpha$ were present in significant quantities, whereas oestradiol-sulfate was present only in trace amounts and oestradiol 17-$\beta$ was undectable. There was no significant difference between the subepithelial stromal and the deep stromal layer of the cervix, with respect to total oestrogen concentrations. For this reason, the data from these two layers were pooled for further analysis. The mean total oestrogen concentrations (pg/g wet weight) in the vaginal, mid and uterine segments of the cervices of the LP4 group (332 ± 47, 434 ± 51, 654 ± 51: the numbers of samples used for each site are depicted in Fig. 1) were significantly higher than those in the equivalent segments of the HP4 group (149 ± 39, 159 ± 41, 138 ± 49: the numbers of samples used for each site are depicted in Fig. 1). within the LP4 group the mean total oestrogen concentrations in the uterine segment of the cervix of was significantly higher than that in mid segment ($p < 0.05$) or the vaginal segment ($p < 0.01$, Fig. 1).

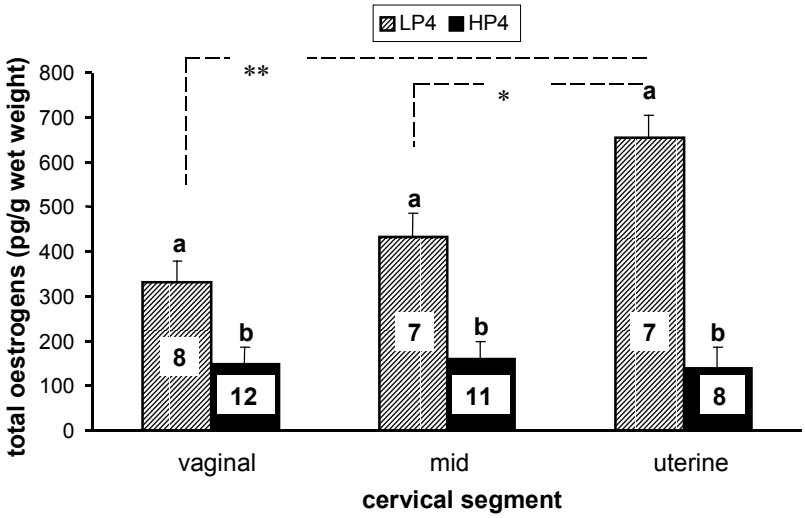


Fig. 1. Mean (LSM ± sem) tissue oestrogen concentrations in the three longitudinal segments of the cervix in non-pregnant cows (pooled values of superficial and deep stromal layers). Within a segment, means with different superscripts differ significantly ($P < 0.01$). Asterisks indicate significant differences (* $P < 0.05$ and ** $P < 0.01$), between the cervical segments in LP4 cows. The numbers depicted in the bars represent the number of samples analysed for each segment of the cervix.

*Immunolocalisation of ER and PR*

Immunohistochemistry demonstrated that ERα were present exclusively in the cell nuclei. PR was also confined to the nuclei. Both ER and PR were present in the luminal epithelial cells, the epithelial crypts and in the fibroblasts and smooth muscle cells of all tissue layers. Vascular endothelium and smooth muscle cells in the walls of blood vessels did not stain positively for either receptor.

*Proportional scores of oestrogen and progesterone receptors*

The progesterone status of the cows (HP4 or LP4) did not significantly affect the proportion of cells that contained ERαs (the proportional score for ERαs). However, there was a significant effect of cervical segment ($p < 0.01$) and tissue layer ($p < 0.01$) on the proportional score for ERαs in both P4 groups. And, since there were no differences between the LP4 and HP4 animals, the values from both groups were pooled to examine the overall effects of segments and tissue layer on the proportional score for ERαs ($n = 10$, sem = 16). In this respect, the mean numbers of ER immunoreactive nuclei per 1000 cells in the vaginal and mid segments were significantly higher than that in the uterine segment ($p < 0.01$, Fig.

2A). In addition, the mean proportional score for ERαs in the deep stromal layer was significantly higher than the score in the subepithelial stroma ($p < 0.05$) and epithelium ($p < 0.01$), and the score in the subepithelial stroma was significantly higher than the score in the epithelium ($p < 0.01$, Fig. 2B).

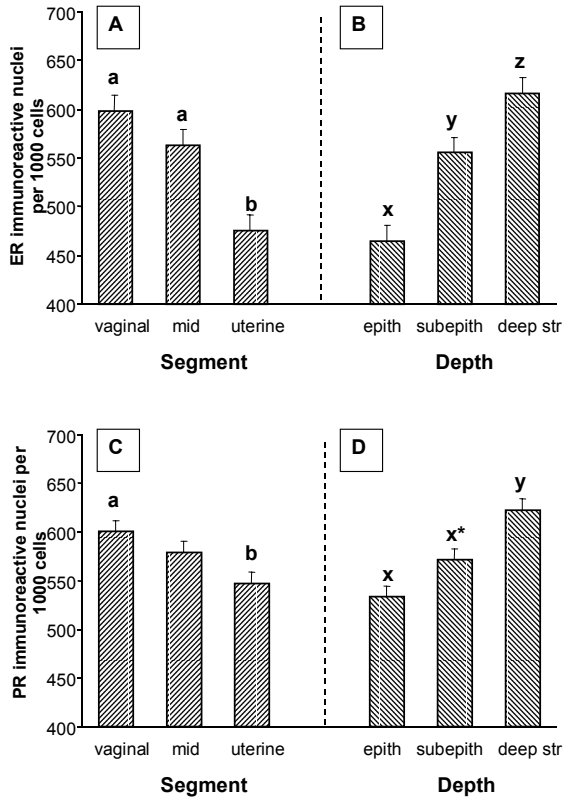


Fig. 2. Mean (LSM ± sem) ER (**A, B**) and PR (**C, D**) immunoreactive nuclei per 1000 cells in the cervix of non-pregnant cows; pooled values for LP4 (n = 4) and HP4 (n = 6) animals (LSM ± sem, n = 10). **A**) Relationship between the proportion of ERα immunoreactive nuclei and cervical segment. Means with different superscripts differ significantly ($P < 0.01$). **B**) Relationship between proportion of ERα immunoreactive nuclei and tissue layer. Differences between **x** and **y** and between **x** and **z** are significant at the $P < 0.01$ level, while the difference between **y** and **z** is significant at the $P < 0.05$ level. **C**) Relationship between proportion of PR immunoreactive nuclei and segment. Means with different superscripts differ significantly ($P < 0.01$). **D**) Relation between proportion PR immunoreactive nuclei and tissue layer. Differences between **x** and **y** are significant at the $P < 0.01$ level, while * indicates that **x*** tends ($P = 0.061$) to be higher than **x**.

Progesterone status (HP4 or LP4) similarly had no effect on the proportional scores for PRs. Again, there was a significant effect of cervical segment ($p < 0.05$) and layer ($p < 0.01$) on the proportion of cells containing PRs in both groups, so that the values of the LP4 and HP4 could be pooled to examine the overall effect of layer and segment on the proportional scores (n = 10, sem = 11). This examination demonstrated that the mean number of PR immunoreactive nuclei per 1000 cells decreased successively from the vaginal segment to the mid segment to the uterine segment, with the difference between the vaginal and uterine segments reaching statistical significance ($p < 0.01$, Fig. 2C). Furthermore, the mean proportional score for PRs in the deep stromal layer was significantly higher than that in the subepithelial stroma ($p < 0.01$) or epithelium ($p < 0.01$, Fig. 2D), while the subepithelial layer tended ($p = 0.061$) to have a higher proportion of PR containing cells than the epithelium.

Additionally, since the different tissue layers in the cervix are thought to have different functions, they were analysed separately to determine if progesterone status affected the proportion of cells containing ER$\alpha$ or PR in any one individual layer. However, there was no significant effect of progesterone status on the proportional scores for either ER$\alpha$ or PR in any of the layers. On the other hand, there was a significant effect of segment on the proportional scores for ERs in the deep stromal and subepithelial layers ($p < 0.05$) and in the epithelial layer ($p < 0.01$). In the epithelial layer, the proportional score for ER$\alpha$s at the vaginal side was significantly higher than at the uterine side (528 vs. 363, sem = 31; $P < 0.05$). In the subepithelial layer similarly, the proportional score for ER$\alpha$s was significantly higher at the vaginal side than at the uterine side (593 vs. 503, sem =19; $P < 0.05$). Finally, in the deep stromal layer the ER$\alpha$ proportional scores in the vaginal and mid segments were significantly higher than that in the uterine segment (674 and 628 vs. 557, sem =27; $p < 0.05$: data not illustrated).

*Cell density and number of ER and PR positive nuclei per mm$^2$*

The ratio between the number of cells in the subepithelial layer and the deep layer varied between 0.41 and 0.55 in the different segments, but there was no significant effect of progesterone status or cervical segment on these ratios. Neither could a relationship be found between the mean cell density and the progesterone status within each of the two layers. Since there was no effect of P4 status, the values of LP4 and HP4 were pooled. These pooled data revealed a significant effect of segment ($p < 0.01$) and tissue layer ($p < 0.01$) on cell density. In the subepithelial layer, there were significantly more cells per mm$^2$ in the uterine segment than in the vaginal segment (114 vs. 87, sem = 14; $p < 0.01$), and the same was true for the deep stromal layer (283 vs. 207, sem = 14, $p < 0.01$). Furthermore, in all 3 segments, the deep stromal layer had significantly more cells per mm$^2$ than the subepithelial stroma (V: 207 vs. 87; M: 223 vs. 110; U: 283 vs. 114; sem = 14; $p < 0.01$, data not illustrated).

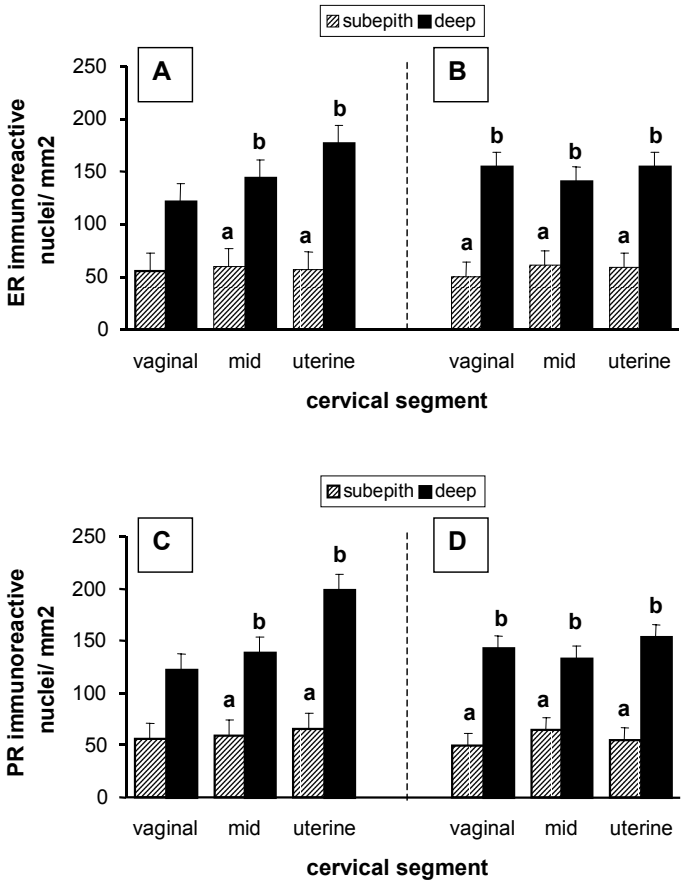


Fig. 3. **A-D**) Mean (LSM ± sem) receptor positive nuclei per $mm^2$ in the superficial and deep stromal layers of three longitudinal segments of the cervix. Within a segment, means with different superscripts are significantly ($P < 0.01$) different. **A**) ER immunoreactive nuclei per $mm^2$ in the LP4 (n = 4) and **B**) the HP4 (n = 6) group. **C**) PR immunoreactive nuclei per $mm^2$ in the LP4 (n = 4) and **D**) the HP4 (n = 6) group.

When the numbers of ERα or PR positive nuclei were expressed per $mm^2$ of tissue, rather than per 1000 cells, differences in receptor expression between the subepithelial and deep stromal layer were more evident, but differences along the longitudinal axis (between segments) disappeared (see Fig. 3A-D). In the LP4 group, the ERα per $mm^2$ in the deep stromal layer at the uterine and mid segments was significantly higher ($p < 0.01$) than in the subepithelial stromal layer (Fig. 3A)

whereas in the HP4 group, there was a significant difference ($p < 0.01$) between the two layers at all three segments (Fig. 3B). The PR per $mm^2$ showed the same pattern as the ER$\alpha$ per $mm^2$ with respect to the effects of tissue layer, cervical segment and progesterone status (Fig. 3C and 3D).

No significant correlations were found between the local oestrogen concentrations in the different tissue sites and either ER per $mm^2$ or PR per $mm^2$. In addition, there was no correlation between receptor expression (ER per $mm^2$ and PR per $mm^2$) and plasma P4 concentrations. When the LP4 and HP4 groups were pooled, the ER$\alpha$ per $mm^2$ showed a strong positive correlation with the PR per $mm^2$ in both layers (subepithelial layer, $R = 0.85$, $p< 0.01$; deep stromal layer, $R = 0.83$, $p < 0.01$).

## Discussion

Given the relative length of the dioestrous and oestrous period in the cycle of cows, the HP4 group was almost certainly more heterogeneous with regard to the stage of the oestrous cycle than the LP4 group. Nevertheless, the significant differences that were found with respect to local tissue concentrations of oestrogens, provide evidence that the LP4 ($\leq$ 0.5 ng/ml) and the HP4 (> 2.0 ng/ml) groups of animals differed markedly from each other in endocrinological respect. Interestingly, this study demonstrated a change in the immunoreactive expression of ER and PR that depended on the depth of the cervical tissue (from epithelium to deep stroma) and thus resembled closely the gradient reported for ER and PR expression in the non-pregnant bovine uterus (Boos *et al*., 1996). This is in contrast with previous authors who did not report this gradient, in spite of differences in the expression of ER and PR between the different tissue layers within the cervix (Kuprya□czyk and Möller, 1988; Cano *et al*., 1990; Snijders *et al*., 1992; Zhao *et al*., 1999). In addition, we demonstrated an increase in the proportion of nuclei per 1000 cells expressing both ER and PR from the uterine to the vaginal end of the cervix. At least in the case of ER, similar differences in expression along the longitudinal axis of the cervix have been reported for women (Kuprya□czyk and Möller 1988; Cano *et al*., 1990). Furthermore, the current study demonstrated that the subepithelial stromal layer of the bovine cervix contains significantly fewer cells per $mm^2$ than the deep stroma, a difference that magnifies the differences in receptor expression between the two layers and, which may reflect a difference in function. This latter observation supports our earlier report that the cervical mucosa is divided into two layers that differ from each other in several morphological and functional aspects, including cell composition and collagen characteristics (Breeveld-Dwarkasing *et al*., 2000, and chapter 2). When receptor expression in the tissue was corrected for cell density, longitudinal differences (uterine to vaginal segment) in receptor expression disappeared. Cosequently, the bovine cervix has characteristic differences in cell density along the longitudinal axis, not differences in ER$\alpha$ or PR density perse. On the other

hand, the increasing gradient of tissue concentration of oestrogens, from vaginal to uterine side of the cervix, indicates that oestrogens may be more active at the uterine end than at the vaginal end.

Against expectations, the current study revealed no apparent effects of systemic progesterone or local tissue oestrogen concentrations on the expression of either ERα or PR in the bovine cervix. This finding agrees with the reports that immunohistochemical ER expression in the cervix of women does not vary during the menstrual cycle (Kuprya□zcyk and Möller, 1988; Cano *et al*., 1990; Snijders *et al*., 1992) and that cyclical variations in the expression of PR are minor (Kuprya□zcyk, 1991; Snijders *et al*., 1992) or non-existent (Cano *et al*., 1990). On the other hand, rather different results were reported for the cervix of cows (Vesanen *et al*., 1991) and mares (Re *et al*., 1995), when cytosolic ER and PR concentrations were measured biochemically, using a dextran-coated charcoal extraction method. In both cases, medium to high serum progesterone levels reduced cytosolic PR concentrations but did not affect cytosolic ER concentrations. The differences between their results and our study may be explained by the fact that these authors did not take into account, longitudinal and cross-sectional differences in ER and PR expression in the cervix. Further more, the two techniques, immunohistochemistry and biochemistry measure receptor expression in very different ways.

The results of the present study also differ markedly from the reports of an almost complete absence of ER and PR immunoreactivity in the mucosal part of the cervix of sheep beyond days 0 to 3 of the oestrous cycle (Zhao *et al*., 1999) and the changes in receptor expression reported during the oestrous cycle of the dog (Vermeirsch *et al*., 1999, Vermeirsch *et al*., 2000) and rat (Wang *et al*., 2000). The irreversible effect of long term exposure to phytoestrogens on the cervical histology in sheep, that does not occur in cattle (Adams, 1995), may indicate very different regulatory mechanisms for steroid receptor expression between the two species, and may be a reason for the differences in the relationship between the stage of the oestrous cycle and receptor expression. Although speculative, the occurrence of a physiological anoestrous, in both dog and sheep, but not dairy cows, may also explain the differences in cyclical changes in receptor expression between the former two species and the cow.

Oestrogen stimulates an increased secretory activity in human cervical epithelium and it is suggested that this effect is mediated by ER (Gorodeski, 1998). In the current study, all cervices from LP4 cows showed gross and histological signs of a highly secretory epithelium and it was, therefore, a surprise to find no significant differences in ER population between the cervical epithelium of the LP4 and HP4 groups. Moreover, the lack of a cycle stage effect on the epithelial ER population is in contrast to the findings of cyclical changes in ERα expression in the epithelium of the rat cervix (Wang *et al*., 2000). However, these latter authors performed their study on the vaginal part of the rat cervix, which has a stratified squamous epithelium that is markedly different to the columnar epithelium of the bovine cervix in the vaginal segment and thus may be subject to different hormonal

regulation. It is also possible that epithelial functions could be controlled in a paracrine fashion via the action of steroid hormones on receptors located in stromal cells, as has been shown for the progesterone receptor in the mouse uterus (Kurita *et al*., 2000a; Kurita *et al*., 2000b).

It is also important to realise that qualitative descriptions of staining patterns or semi-quantitative scoring methods based on a combination of the intensity of the staining reaction and the number of stained cells, as used in some of the previous studies, provide no indication of the amount of receptor protein present in the nucleus. Furthermore, since both steroid-occupied and steroid-unoccupied forms of the receptors are recognised by the antibodies (Press and Greene, 1988), immunopositive staining of the nucleus does not itself necessarily reflect the level of hormone or receptor activity. Of course, it is possible that the effect of the steroid hormones on the cyclical changes in the physiology of the cervix, are not regulated simply and exclusively by receptor concentration. For example, oestrogens are thought to be able to induce non-genomic reactions in the absence of the ER (Lueng and Wathes, 2000). Furthermore, endogenous suppressers of estrogen activity exist, that in competition with co-activators decrease the transcriptional activity of the ER (Delage-Mourroux *et al*., 2000). The tissue specific effects of oestrogen may thus be influenced by tissue and cell specific patterns of co-activators and co-suppressers of ER (reviewed by Klinge, 2000). The ER antibody used in the present study was directed against ER$\alpha$ and, although it was not always explicitely stated, that was probably the case in most of the other reports cited. Interestingly, the effects of oestradiol-17$\beta$ binding to ER$\beta$ can oppose those of ER$\alpha$ binding (Paech *et al*., 1997) and this suggests that the balance of the different receptor isoforms may affect the biological effect of the ligand. Our present knowledge on the distribution of both ER isoforms in the cervix of non-pregnant individuals of the different species is limited. In rats, it has been reported that ER$\alpha$ and ER$\beta$ are distributed equally within the cervix and, their expression changes in a parallel fashion during the oestrous cycle, despite much weaker ER$\beta$ expression (Wang *et al.* 2000). Also, in the rat the immunohistochemically detected expression of both oestrogen receptor isoforms differs with respect to the region of the cervix (Wang *et al*., 2000).

In summary, our immunohistochemical study demonstrated the absence of any oestrous cycle related fluctuations in ER and PR expression in the bovine cervix. It also demonstrated the presence of a significant relationship between receptor expression and the depth of the tissue layer (from epithelium to deep stroma), which suggests that the effects of steroid hormones on receptor expression are regulated at a regional level, although paracrine influence from nearby tissue layers can not be ruled out. The earlier observation that the relative proportions of smooth muscle and fibroblast cells in the subepithelial and deep stromal layers are different (Breeveld-Dwarkasing *et al*., 2000), may help to explain differing ER and PR concentrations in the two layers and further suggests that the effect of hormonal stimulation is likely to be different between the two layers.

Correcting receptor expression values for cell density further emphasised the differences between the subepithelial and deep stromal layers. On the other hand, correction for cell density removed any differences in receptor expression between the vaginal and the uterine segments of the cervix. In conclusion, these results demonstrate that oestrogen and progesterone receptor expression in the bovine cervix is not controlled simply by circulating steroid hormone concentrations. Furthermore, the regional differences in receptor expression described, demonstrate clearly that great care is needed in the selection of tissue before conclusions can be made with regard to the effects of circulating hormones on receptor expression.

## Acknowledgements

The authors thank Dr. T.A.E. Stout, for his critical reading of the manuscript.

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**-Chapter IV-**

# Changes in water content, collagen degradation, collagen content and concentration in repeated biopsies of the cervix of pregnant cows

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-submitted for publication-

## Abstract

The objective of this study was to assess if cervical ripeness could be quantified by measuring the percentage of denaturation of the collagen network of the stromal layer. Biopsies from the caudal part of the cervix were obtained from 9 pluriparous cows between day 149 and 157 of gestation (second trimester biopsy), at exactly day 275 of gestation (term biopsy) and shortly after calving (calving biopsy). The samples were divided into a superficial stromal part and a deep stromal part. The water content was derived from the weight of the samples before and after lyophilisation. A colorimetric assay was used to assess the percentage of collagen denaturation by determining the extinction at 570 nm of hydroxyproline released from $\alpha$-chymotripsine treated samples. By incorporating a hydroxyproline standard series in the measurements, the collagen content ($\mu$g per mg dry weight) as well as the collagen concentration ($\mu$g per mg wet weight) could be derived.

The water content of both layers of the cervix, significantly increased between mid pregnancy and parturition ($p < 0.01$). The collagen content and the collagen concentration were significantly increased at term ($p < 0.01$ and $p < 0.05$, respectively), but significantly decreased at calving ($p < 0.05$ and $p < 0.01$, respectively) Both parameters showed no significant differences between the superficial and deep stromal layer and they were significantly correlated with each other. A significant increase in the percentage denaturation of the deep stromal layer occurred between the second trimester and term pregnancy ($p < 0.01$), while at calving the percentage denaturation had not significantly increased compared to term. The percentage of collagen denaturation of the superficial stromal layer did not significantly change with stage of gestation or at parturition.

Our findings indicate that cervical ripening is a combination of increased collagen synthesis, and an increased percentage of collagen denaturation, while at calving an increased digestion of the denatured collagen leads to increased collagen loss from the cervical connective tissue. The finding that cervical ripening mainly takes place in the deep stromal layer of the cervix, emphasises the importance of a detailed description of the tissue sampling sites for a proper interpretation of the results obtained from biochemical studies of the cervix.

## Introduction

During the largest part of gestation, the cervix is stiff and non-stretchable to keep the foetus safely isolated in the uterus. Extensive remodelling of the connective tissue is necessary to enable the cervix to dilate at the time of parturition. Collagen is one of the major components of the cervical connective tissue, and because of its cross-linked three dimensional structure, it contributes greatly to the stiffness of the cervix. Since the first reports on the fibrous nature of the cervix (Danfort, 1947), there have been many studies on the changes in the connective tissue during pregnancy and parturition. However, in spite of the advances in biochemistry and molecular biology there is still no uniformity in the data on cellular and biochemical changes in the cervical connective tissue (Winkler and Rath, 1999). In most species that have been studied until now, ethical and anatomical restrictions make it almost impossible to obtain repeated biopsy samples of the cervix during different stages of pregnancy. Differences in sampling procedures, insufficient detailing of the sampling site and differences in analysing techniques, complicate the interpretation of many reports on structural changes in the cervical tissue (Leppert, 1995, Winkler and Rath, 1999). For example, in sheep (Fitzpatrick, 1977) and rats (Shi *et al*., 1999), decreasing collagen concentrations (related to the wet weight) have been reported during the last trimester of gestation without any further decrease at parturition. However, for the cow it has been reported that while the collagen concentration of the cervix decreases towards the end of gestation, the total collagen content (related to the dry weight) at the same time increases (Kaidi *et al*., 1995) and a similar report was made for rats (Harkness and Harkness, 1959). It has also been suggested that the decrease in cervical collagen concentration is the result of the simultaneously increasing content of water and of other cervical proteins, such as proteoglycans (Leppert, 1995).

Changing of the collagen structure starts already at the beginning of the third trimester of gestation, as has been shown in several species such as the rat (Harkness and Harkness, 1959, Rimmer, 1973, Shi et al., 1999), sheep (Fitzpatrick, 1977) and cow (Kaidi *et al*., 1995). However, cervical softening or ripening, which results from the collagen fibres becoming dispersed and less aggregated compared to those of the non-pregnant cervix, does not necessarily coincide with the loss of tensile strength (Yu *et al*., 1995). Microscopic and electron-microscopic studies have shown that in the collagen network of the ripened pregnant cervix, the dense fibre alignment has changed into a partly degraded, loosely organised network with shorter fibres (Kleisl et al., 1978, Parry and Elwood, 1981; Leppert, 1995), that can be more easily extracted from the tissue (Uldjberg *et al*., 1983; Granström *et al*., 1989). Because intact triple helical collagen molecules are highy resistant to proteolytic enzymes, whereas denatured (unwound) collagen is easily digested, and therefore easily extracted from the tissue (Bank *et al*., 1997), we hypothesised that, the percentage of denaturation of the collagen network may serve as a quantitative measure of cervical ripeness. This was investigated in the present study. In a previous experiment in non-pregnant cycling cows, we demonstrated that regional differences in collagen

biochemistry exist along the circular axis of the cervix (chapter 2). Therefore, we performed our measurements in both the superficial stromal layer and the deep stromal layer of serial biopsies obtained from the caudal cervix of cows 1) halfway the second trimester 2) at day 275 of gestation (term) and 3) shortly after spontaneous calving. In addition to the percentage of collagen denaturation, we also assessed the collagen content as well as the collagen concentration, and the water content in the two stromal layers of these biopsy specimens.

## Materials and Methods

*Reagents*

Guanidium chloride (GuHCl), EDTA, iodoacetamide and α-Chymotripsine (αCT) were obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid 37% (or 12M), sodium acetate trihydrate, sodium hydroxide p.a., acidic acid, 2-propanol, chloramine-T, dimethylaminobenzoaldehyde (DMBA), perchloric acid 60% and hydroxyproline were obtained from Merck (Darmstadt, Germany) and citric acid was obtained from Fluka (Buchs, Switzerland).

Incubation buffer consisted of 1mM iodoacetamide and 1mM EDTA in PBS, pH 7.5. A solvent of 4M GuHCl in incubation buffer was used for extraction of proteoglycans and soluble collagen. Digestion buffer was made by dissolving 1mg/ ml αCT in incubation buffer. Stock buffer, pH 6.1 contained 50.44 g/ l citric acid, 117.76 g/ l sodium acetate trihydrate and 34 g/ l sodium hydroxide p.a. Assay buffer was made by mixing 100 ml stock buffer with 30 ml 2-propanol and 20 ml de-ionized water. Chloramine-T reagens contained 0.141g chloramine-T dissolved in 1ml 2-propanol, 1 ml de-ionized water and 8 ml stock buffer. DMBA reagens contained 4 g DMBA in 2.5 ml 2-propanol and 5.5 ml 60% perchloric acid. The 200 μM hydroxyproline standard contained 26.23 μg/ ml hydroxyproline.

*Animals and Collection of samples*

Cervical biopsies were collected from 9 Holstein Friesian or Holstein Friesian/Dutch Friesian crossbred, pluriparous cows, at the two different stages of gestation and at calving between January and November 1999. The cows were housed at the experimental farm of the Faculty of Veterinary Medicine of the Utrecht University and belonged to the commercially kept, high yielding dairy herd. A dry-of period of 8 weeks before the expected day of parturition was a standard procedure at the farm. The experimental procedure was approved by the Committee for the use of animals in research of the Utrecht University. The cows were fed according to their individual nutritional needs as defined by their level of milk production and stage of gestation. Cervical biopsies were obtained transvaginally, by using a skin biopsy punch of 6 mm diameter (Kai industries co. Ltd. Oyana, Japan). The biopsies were collected at the inside of the cervical canal, approximately 2 cm cranial to the vaginal cervical opening. Care was taken that the deep stromal layer was always included in the sample. The first biopsy was

collected between day 149 and 157 of gestation (second trimester biopsy), the second one at 275 days of gestation (term biopsy) and the third within two hours after spontaneous calving (calving biopsy). The day of artificial insemination was day 1 of gestation. Prior to collection of a biopsy, two and a half ml of Lidocaine HCl 2% (Apharmo, Arnhem, The Netherlands) was applied in the first intercoccygeal area as an epidural anaesthesia, to avoid abdominal straining during the vaginal manipulations. Immediately after obtaining the biopsies, they were cleaned from mucus and blood. The outer muscle layer was removed and the cervical stromal tissue was divided into a superficial part, containing the epithelium and soft fibrous tissue directly underneath, and a deep part, containing the more peripheral fibrous tissue. The samples were then snap frozen in liquid nitrogen, and stored at - 80°C until further analysis.

In addition, venous blood samples were collected from the tail vein, the days that the cervical biopsies were collected, to measure plasma progesterone concentrations by means of a validated direct solid phase $^{125}$I RIA (Dieleman and Bevers, 1987) with a sensitivity of 47 pg/ ml, an intra-assay coefficient of variation of 11% (n = 16) and an intra-assay coefficient of variation of 7.5% (n = 20).

*Water content*

After thawing and rinsing the samples with isotonic saline, they were lyophilised for 24 h. The water content of the samples was derived from the weight of the samples before and after lyophilisation. The wet weight of the samples varied between 105 mg and 1579 mg, and the dry weight varied between 11 mg and 246 mg.

*Analysis of collagen content, collagen concentration and denaturation*

After lyophilisation, the samples were rehydrated in isotonic saline. Proteoglycans and soluble, non-crosslinked collagen were extracted from the samples by washing them 6 times with 1ml 4M GuHCl in incubation buffer, at 4°C for 48 h. The tissue samples were then washed 3 times for 6 h at 4 °C with 1 ml incubation buffer to remove the GuHCl. After this, they were digested for 24 h at 37°C with 1 ml digestion buffer. The supernatant, containing the αCT-solubilized collagen fragment was removed and 500 μl was diluted 1:1 with 12 M HCl. The remaining tissue was immersed in 800 μl 6M HCl after which supernatant and remaining tissue were hydrolysed at 110 °C for 24 h and dried. The dried hydrolysates were dissolved in 1.5 ml of deionized water. The hydroxyproline (Hyp) concentration of the hydrolysates from the supernatant and the remaining tissue was measured by a colorimetric method according to the principles of Stegeman and Stadler (1967) and as described by Creemers et al, (1997). In short, the dissolved hydrolisates of the supernatant and of the remaining tissue were diluted 6 fold. From these diluted samples, 60 μl was pipetted into a well of a polystyrene microtiter plate (Greiner 655101, Frickenhausen, Germany), after which 20 μl assay-buffer and 40 μl chloramine-T reagens were added. After a 20 min incubation at room temperature, 80 μl DMBA reagens was added to the samples,

and carefully mixed. Subsequently, the plate was closed with a lid, placed in a small water bath, which was then placed in an incubator at 60 °C for 25 minutes. The plate was cooled down by placing it in a water bath containing cold water for 5 min, during which time the water was refreshed once. Subsequently, the extinction was measured at 570 nm on a Titertek multiscan MCC/340 (Labsystems, Finland). A hydroxyproline standard series (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 μM) and blancs (water) were included in the measurements. The extinction measured for the standards and the samples was corrected for the blancs. Samples that had an extinction higher than that of the maximum dilution of the standard series were diluted further and measured again.

The hydroxyproline concentration of the hydrolysed samples was interpolated from the standard curve. It was assumed that collagen contains 300 hydroxyproline residues per triple helix and that the molecular weight of collagen is 300 kDA and therefore, the collagen content (μg) of the cervical biopsy samples could be calculated from the hydroxyproline concentration of the supernatant and the tissue hydrolysates. The collagen content was calculated by expressing collagen relative to the dry weight of the cervical tissue, whereas the collagen concentration is expressed relative to the wet weight of the tissue.

The percentage collagen denaturation of the samples was calculated with the following formula:

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

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.

*Statistical analysis*

Results are expressed as means ± sem. A General Linear Model (GLM) procedure (SAS, 1990) was used to analyse the data with the following model:

$$Y_{ij} = \mu + G_i + e1_i + Depth_j + e2_{ij}$$

The model tested against differences between depth of the tissue layers and between gestational age, against variation within individual cows. $Y_{ij}$ = dependent variable; $\mu$ = overall mean; $G_i$ = gestational age (i = second trimester, term or calving); $e1_i$ = error term, which represents the inter-cow variation; $Depth_j$ = depth of the tissue layer (j = superficial, deep) and $e2_{ij}$ = residual error. After testing all the interactions, significant effects were re-analysed in more detail. Significance was accepted at the $p < 0.05$ level. Pearsons correlation test was used to analyse the relationship between the different parameters.

## Results

*Progesterone levels and calving results*

All 9 cows calved spontaneously and delivered healthy calves. The gestational age at calving varied from 277 to 292 days, which means that the interval between the term biopsies and the calving biopsies varied from 2 to 17 days. Plasma P4 concentrations in the individual cows ranged from 7.6 to 5.0 ng/ ml at first (= second trimester) sampling. On day 275 of gestation (second biopsy), they ranged from 7.7 to 1.0 ng/ ml, while at calving (third biopsy), the levels ranged from 0.10 to 0.04 ng/ ml. The plasma P4 concentrations were significantly related with gestational age ($p < 0.0001$).

*Water content*

A significant effect of the gestational age ($p < 0.0001$) and of the depth of the tissue layer ($p < 0.01$) was found on the water content of the cervix (Fig. 1). Within the same gestational age, there were no significant differences in water content between the superficial and the deep stromal layer. Within one layer (superficial or deep), the water content increased significantly with gestational age. The water content of the superficial stromal layer increased significantly between the second trimester and term and between term and calving ($p < 0.01$). Similarly, the water content of the deep stromal layer increased significantly from second trimester to term and calving ($p < 0.01$).

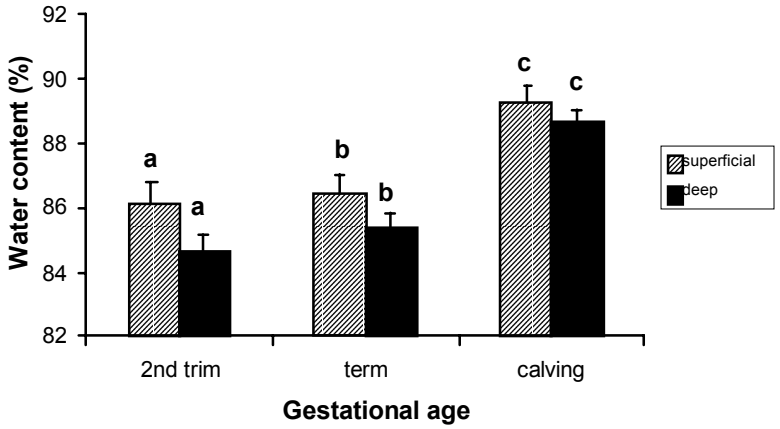


Fig. 1. The water content (% of wet weight, means ± sem, n = 9) of the superficial and deep stromal layer of the bovine cervix, during three gestational ages. Different letters within one layer indicate significant differences between gestational ages ($p < 0.01$).

*Collagen content and Collagen concentration*

The collagen content and the collagen concentration of the cervix ($p < 0.001$) were significantly influenced by gestational age, without any effect of the depth of the tissue layer. Therefore, the values of the superficial stromal layer and the deep stromal layer were pooled, for further statistical analysis of the effect of gestational age on both parameters. Between the second trimester and term, the mean collagen content of the cervix increased significantly ($p < 0.01$) by 36%, while at calving, it had returned to its second trimester value (Fig. 2A). The collagen concentration of the cervix also increased significantly between second trimester and term, by 23 % of its second trimester value ($p < 0.05$) and decreased between term and calving to values not different from that in the second trimester (Fig. 2B). The collagen content and collagen concentration were positively correlated ($R = 0.8$, $p < 0.0001$) with each other.

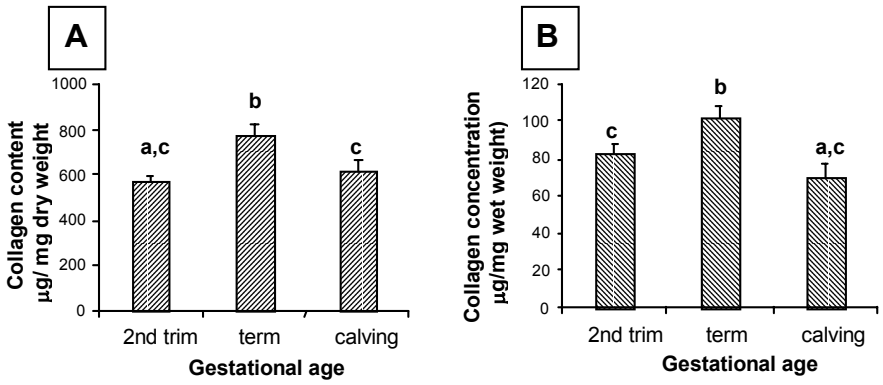


Fig. 2. **A**) mean collagen content (µg/ mg dry weight, ± sem, n = 9) and **B**) mean collagen concentration (µg/ mg wet weight, ± sem, n = 9) of the bovine cervix (pooled data from superficial and deep stromal layer) during the two gestational ages and at calving. Different letters indicate significant differences between the different gestational ages (a – b, $p < 0.01$; b – c, $p < 0.05$).

*Percentage denaturation of collagen*

The percentage of collagen denaturation in the cervix was significantly affected by gestational age ($p < 0.0001$) and depth of the tissue layer ($p < 0.001$) (Fig. 3). There was a significant increase in percentage denaturation in the deep stromal layer from the second trimester to term ($p < 0.05$), but not between samples taken at term and at calving. During the second trimester, the percentage of collagen denaturation in the superficial stromal layer was significantly higher ($p <$

0.05) than in the deep stromal layer, while during the other two stages the differences between the two layers was non-significant (Fig. 3).

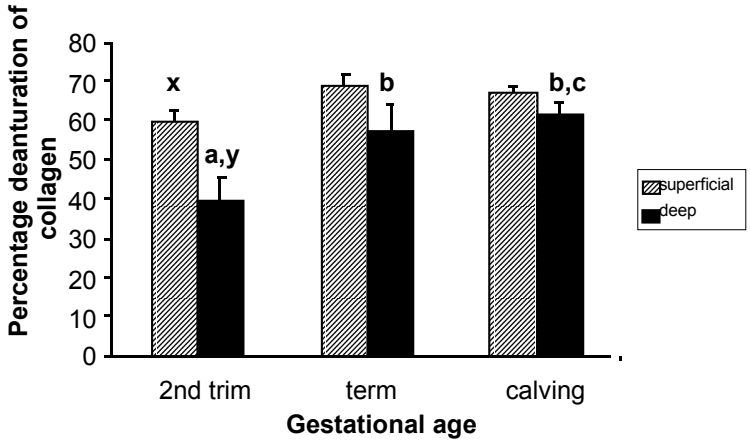


Fig. 3. Mean percentage denaturation (± sem, n = 9) of the superficial and deep stromal layer of the bovine cervix, at two gestational ages an at calving. Different letters indicate significant differences between layers (x - y, $p < 0.05$) or between gestational ages (a – b, $p < 0.05$; a – c, $p < 0.01$).

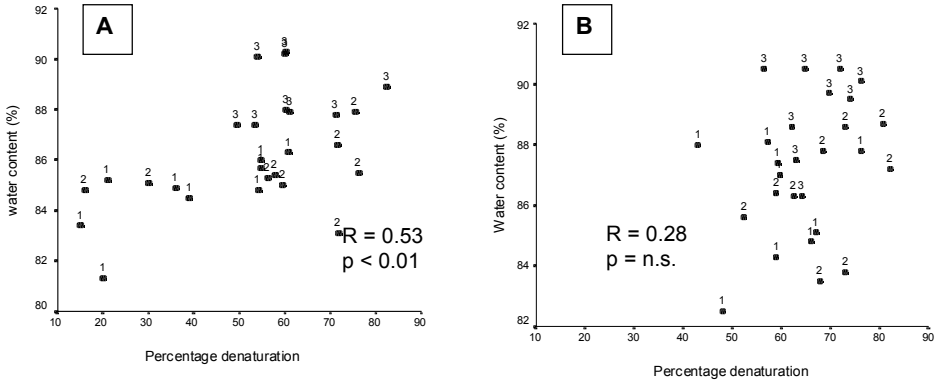


Fig. 4. Correlation between the percentage denaturation of collagen and the water content of **A**) the deep stromal layer and **B**) the superficial stromal layer of the bovine cervix. The numbers denote the samples obtained at the three different gestational ages; 1 = second trimester, 2 = term, 3 = calving. The correlation is significant in the deep stromal layer but not in the superficial stromal layer.

Overall, there was a positive, but weak correlation between the percentage denaturation and the water content ($R = 0.46$, $p < 0.001$), that was, however, only significant in the deep stromal layer ($R = 0.53$, $p < 0.01$, Fig 4A and B). In the deep stromal layer the increase in water content between second trimester and calving was correlated with the increase in percentage denaturation ($R = 0.70$, $p < 0.05$, data not shown).

## Discussion

We found an increase in cervical collagen content and concentration and an already maximally increased percentage of collagen denaturation at term pregnancy, even before calving. These findings indicate that cervical ripening is a gestational process during which an increased collagen synthesis is accompanied by a significant increase in the percentage of collagen denaturation, while the degraded collagen molecules remain incorporated in the collagen network of the tissue. This degraded collagen is more susceptible to digestion by proteinases (Bank *et al*., 1997).

Our results also demonstrate that collagen degradation in the deep stromal layer of the bovine cervix at term is already significantly increased compared to the second trimester, and from there on it does not increase until calving. During the second trimester, the percentage of collagen denaturation in the superficial and deep stromal layer were much higher than values we found during the non-pregnant state (chapter 2). But, similar to the cervix of non-pregnant cows (chapter 2), significant differences in percentage of denaturation were found between both stromal layers of the cervix. The observation that such differences are no longer apparent during term pregnancy and calving, and that the percentage denaturation in the superficial layer did not increase significantly during the three sampling moments, indicate that cervical ripening mainly takes place in the deep stromal layer. This is in accordance with the data of Yu *et al*. (1995) in the rat, who described three distinct regions along the cross sectional axis and found that during cervical softening, the microscopic loss of structure in the middle layer of the cervix was more significant than in the other two.

In the present study both the collagen content and the collagen concentration of the cervix had been significantly increased between second trimester and term, but both had dropped again significantly at parturition. These results are not in agreement with those of others who reported that collagen decreased from early pregnancy to term pregnancy and that no further change occurred between term and labour in women (Danfort and Buckingham, 1964; Uldjberg *et al*. 1983; Stjernholm *et al*., 1996; Granström *et al*., 1989), sheep (Fitzpatrick, 1977; Regassa and Noakes, 2001) and cows (Kaidi *et al*., 1995).

Our results also show that throughout gestation a significant correlation exists between the collagen content and the collagen concentration. In most of the studies performed by other groups, collagen was either expressed relative to the dry weight or total protein content of the cervix, or relative to the wet weight.

However, in the case where both parameters were used, it was reported that the total collagen content of the cervix increased during gestation, while the collagen concentration of the cervix gradually decreased (cows: Kaidi *et al*., 1995; sheep: Regassa and Noakes, 2001; rats: Harkness and Harkness, 1957). However, these authors measured the collagen content of the entire cervix and as such, their results reflect the growth of the entire cervix rather than an increase of the collagen content per unit dry weight of connective tissue.

The discrepancy between our data reporting an increase of collagen concentrations between second trimester and term pregnancy and the decrease reported by others, are probably explained by methodological differences. Our values with respect to collagen content and concentration reflect the sum of degraded collagen, still incorporated within the network, plus the intact collagen. By contrast, in at least three of the reports on the cervix of women (Uldjberg *et al*. 1983; Stjernholm *et al*., 1996; Granstrom *et al*., 1989) it has to be assumed that pepsin extraction was performed before hydroxyproline was quantified. This would have digested the denatured collagen, which was then not included in the assessment of the collagen concentration. We conclude from our results, that between second trimester and term pregnancy, cervical ripening involves an increased synthesis of collagen and at the same time an increased rate of collagen degradation. This is in accordance with the claim of Kleisl *et al*. (1978), that partly degraded collagen is retained within the tissue, but it does not support the suggestion made by Leppert *et al*. (1995), that the loss of structure of collagen during ripening is not the result of an increased degradation but rather the result of an increase of loosely bound, newly synthesised collagen.

The decrease in both collagen content and collagen concentration between term pregnancy and calving as found in our study, could be explained by further digestion of the degraded, though still cross-linked, collagen into soluble, non cross-linked collagen fragments. These are easily washed out during the GuHCl extraction. Likewise, newly synthesised collagen fragments that might have been present during al the three sampling periods have also been washed out with the GuHCl. If this had not been done, these fragments would have been included in the $\alpha$CT-supernatant and this would lead to an overestimation of the percentage of collagen denaturation, which in turn could lead to an overestimation of the degradative processes in the tissue.

In this experiment, the water content was significantly increased at term pregnancy compared to the second trimester, but in contrast to the percentage collagen denaturation, it had increased even further at calving. This was true for both the superficial and the deep stromal layers. Although others did not differentiate between the superficial layer and deep layer of the cervix, the increase in water content as measured in this study is of the same magnitude as in women (Uldjberg *et al*., 1983) and rats (Cabrol, 1982). However, again others did not find significant differences in water content with increasing gestational age (cows: Kaidi *et al*., 1995; sheep: Regassa and Noakes, 2001; women: Granstrom *et al*., 1989). Meanwhile, the actual increase in water content is relatively small and can

therefore, hardly explain the histological findings of the widely dispersed collagen fibres at parturition as compared to the more densily packed fibres of the non-pregnant and pregnant (term) cervix (Danfort and Buckingham, 1964). Neither can it explain the weight gain of the cervix as was suggested for the rat by Cabrol *et al*. (1982). Although, part of the decrease in collagen concentration, as found in this study between term pregnancy and calving, may have been caused by the increase in water content of the cervix, the collagen concentration showed a strong, positive correlation with the collagen content, the latter being independent of the water content. This observation favours the explanation already given above, that collagen loss due to increased digestion was responsible for the decrease in both collagen content and concentration. Additional mechanisms, such as an increased content of other proteins, namely glycosaminoglycans, may also be responsible for the decrease in collagen content at calving.

There is also indirect evidence that the regulation of collagenolytic events during cervical ripening differs from that during cervical dilatation. Massive influx of leucocytes occur during active labour in sheep (Owiny *et al*., 1995), women (Yoshida and Manabe, 1990; Knudsen *et al*., 1997; Winkler *et al*., 1999) and rats (Luque *et al*., 1996), while they are almost completely absent during early and term pregnancy. This indicates that different proteinases may be active at the two stages. Some authors report that collagenolytic activity is already increased during term pregnancy (Uldjberg *et al*., 1983; Granström *et al*., 1992) or only during active labour (Rajabi *et al*., 1991; Winkler *et al*., 1999), while others did not find any evidence for increased activity at all (Raynes *et al*., 1988a; Raynes et al., 1988b). However, the assays that were used in these studies were not specific for the different proteases and this may have influenced the interpretation of their results. Different matrix metalloproteinases (MMPs) are known to act on different substrates or on different regions of the same substrate (Cawston, 1996; Murphy and Knäuper, 1997; Chandler *et al*., 1997). For example, collagenase-1 (MMP-1), which is of fibroblast origin is said to preferentially cleave type III collagen, while collagenase-2 (MMP-8), which is of neutrophilic origin is more active against type I collagen (Balbin et al., 1998). Collagenases cleave interstitial collagen triple helices, while gelatinases (MMP-2, -9), another subfamily of MMPs, digest unwound collagen molecules and gelatin (Cawston, 1996; Murphy and Knäuper, 1997; Chandler *et al*., 1997). It is possible that during cervical ripening of late pregnancy, fibroblast-collagenase plays a more active role by cleaving the triple helical structure of the collagen molecule, leading to an increase of collagen denaturation as found in the present study. Subsequently, the neutrophil-collagenase, the gelatinases and possibly other proteolytic enzymes, may further digest the collagen into small extractable fragments during cervical dilatation. Further study is needed to differentiate the actions of the different collagenolytic enzymes, involved in cervical softening and dilatation. The biopsy technique used in the present study, may be a useful tool for such studies.

In none of the cows of this experiment did we observe any clinical illness or premature labour associated with the moment that the biopsies were collected. In

the present study, the gestational age of the pregnancies determined the days of the first two biopsy collections. It should be realised that animals with the same gestational age are not necessarily at the same stage relative to parturition. This is illustrated by the rather large variation of the interval between the term biopsies, that were collected at a fixed gestational age of 275 days, and the biopsies that were taken directly after calving. This might explain at least part of the variation in collagen data between cows. The functional changes in the cervix that lead to the loss of tensile strength as needed to achieve cervical dilatation, most probably start very shortly before or at the moment that parturition is initiated. This calls for a more detailed study of the period between term pregnancy and calving, in which the timing of the collection of biopsies is related to the stage of parturition. In the case of spontaneous calving cows, this would hardly be possible. Therefore, efforts should be made to find a suitable protocol for induction of parturition, which closely mimics the physiological events during calving.

In summary, the results of this study suggest that cervical ripening is a process, that probably takes place already during gestation and that involves both increased collagen synthesis and increased collagen degradation. Cervical dilatation during parturition (labour), on the other hand, is associated with increased collagen loss, most likely as a result of increased proteolytic activity. The findings that characteristics of cervical ripening are more pronounced in the deep stromal layer than in the superficial stromal layer of the cervix, emphasises that a detailed description of the origin of the tissue samples is necessary for the interpretation of the results of studies on cervical ripening and softening.

## Acknowledgements

The authors thank N.M. Soede of the Wageningen University for the statistical analysis of the data.

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**-Chapter V-**

# Ultrasonic cervimetry to study the dilatation of the caudal cervix of the cow at parturition

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Theriogenolgy (2002), 57: 1989-2002

## Abstract

The objective of this study was to investigate the temporal changes in dilatation of the caudal cervix during induced calvings (n=5). We used ultrasound cervimetry, allowing the continuous recording of the distance between a transmitting and receiving ultrasound crystal, which were implanted opposite to each other on the caudal rim of the cervix. We started recording between 19 and 21 h after injecting a prostaglandin analogue (PG) on day 272 of gestation. A fluid-filled catheter had been introduced transcervically between the fetal membranes and the uterine wall for measurements of intra-uterine pressure (IUP). While the characteristics of calving varied widely between the five animals, it appeared possible to divide the process of dilatation into four phases. During the latent phase, which lasted until 25 to 43 h after PG, no net gain in dilatation occurred. We found an acceleration phase (4.3 to 6.8 h), in which the dilatation rate speeds up (0.49 to 0.84 cm/h) in three of the cows. During the phase of maximum slope (lasting 0.5 to 4.8 h), we measured an even higher rate (1.47 to 8.48 cm/h), decreasing again during the deceleration phase (rate 0.24 to 2.28 cm/h) in four cows.

The quality of the IUP measurements precluded us from continuously investigating the relationship between cervical dilatation and uterine contractions. However, short term simultaneous recordings revealed that the cervical opening changed momentarily in the absence of IUP during the latent phase, while during the phase of maximum slope, temporary changes of dilatation coincided with uterine contractions. We concluded that the method of ultrasound cervimetry used in this study provides a valuable way to study the process of cervical dilatation in parturient cows in vivo.

## Introduction

Little is known about the normal dilatation pattern of the bovine uterine cervix during calving and how this might be related to uterine activity. Before unraveling the pathophysiology of the insufficiently dilating cervix in cows, it is important to better understand the dynamics of the cervix during normal parturition. To the best of our knowledge, there is presently only one study that attempted to record the dilatation of the cervix continuously in parturient cows (Gregory, 1977). Using an electromechanical device, a maximal distance of only 7cm dilation could be measured. In addition, there is one author who has constructed a graphical representation (partogram) of cervical dilatation for the cow, combining intrauterine pressure measurements with manually assessed cervical dilatation (Rüsse, 1963).

Not only in the cow, but even more so in almost all other domestic species, there is a lack of information about the dynamics of cervical dilatation during normal parturition. In contrast, in human obstetrics, partograms of the dilatation of the cervix have been made on the basis of digital examinations (Richardson and Sutherland, 1977). One examination that was based on a high number of observations and is still used extensively is the classical Friedman curve (Friedman, 1955; Friedman 1956). Caliper-type or string-type mechanical devices, as well as electromagnetic cervimeters, have also been used in women (Richardson and Sutherland, 1977; van Dessel *et al*., 1991). These were later combined with potentiometers to convert the movements of the caliper arms to an electrical signal that could be recorded continuously on a polygraph to represent the dilatation curve graphically (Richardson and Sutherland, 1977).

In recent years, real time ultrasonography has been used to visualize cervical dilatation through abdominal, vaginal and transperineal routes in women (Okitsu *et al*., 1992; Zilianti *et al*., 1995; Iams *et al*., 1996; Bergella *et al*., 1997; Burger *et al*., 1997; Iams, 1997; Rageth *et al*., 1997;). We can visualize the length of the cervix, the dilatation of the internal os of the cervix and the dilatation of the cervical canal with this method. However, this method is subject to inter- and intra-observer variation (Burger *et al*., 1997). Although it is possible to visualize the bovine cervix during parturition by means of transvaginal scanning (personal observation first author), the necessary repeated vaginal manipulations may affect the physiology of the dilating cervix. In order to study the dilatation of the cervix of the cow longitudinally, a method is needed that allows a continuous measurement, without the risk of artifacts and without discomfort to the animal. Before the development of real time ultrasonography for the visualization of the cervix, ultrasonic techniques were applied in women to record cervical dilation continuously during parturition (Eijskoot *et al*., 1977; Kok *et al*., 1977). With such a method, two ultrasound transducers are placed opposite to each other on the rim of the external os of the cervix. One transducer acts as a transmitter, and the time needed for the ultrasound pulses to reach the opposing receiving-transducer is distance-dependant. Because the propagation velocity of ultrasound in vaginal and cervical tissue is approximately 1500 m/sec and remains constant, we can continuously calculate the distance between the transducers and, thus, the degree

of dilatation of the caudal part of the cervix. By combining this method with the measurements of IUP changes, we can establish the relationship between uterine activity and cervical dilatation (van Dessel *et al*., 1991).

The aim of the present study was to explore the dynamics of cervical dilatation in the parturient cow, and the use of an ultrasound cervimeter, which was adapted from human studies.

## Materials and Methods

*Animals and Instrumentation*

In this experiment, we used pure Holstein Friesian breed or crossbreeds (75% HF/ 25% FH, n = 5). We bought them with known breeding data from the market. Four of them were heifers (primigravid) and one was in her second pregnancy. At least one week before the experiment started we housed the cows in a stable with individual stands, which were subjected to normal daylight. We limited concentrates and provided silage and water ad libitum. We induced parturition on day 272 of pregnancy, with a single intramuscular injection of 2 mL of a synthetic $PGF_{2\alpha}$ analog (Luprostiol, 7.5 mg/mL; Prosolvin®, Intervet International, Boxmeer, The Netherlands) in the hind-leg. At that time, we placed the cow in an individual pen where she remained until calving was completed. We instrumented the cow at approximately 18 h after the $PGF_{2\alpha}$ injection (PG). We administered 2.5 mL lidocaïne cum adrenaline in the first intercoccygeal area for epidural anaesthesia (Lidocaïne HCl 2%®, Apharmo, Arnhem,The Netherlands), to prevent abdominal straining while placing the transducers on the cervix. After taking standard hygienic measures, we manually assessed the location and softness of the portio vaginalis of the cervix. We embedded the ultrasound transducers, with a diameter of 6 mm, in a square epoxy plate (13 × 13 mm) with mounting holes of 1 mm at each corner. We sutured the plates tightly onto the caudal cervical rim, at a 3 o'clock and 9 o'clock position, with an initial distance of 3 to 5 cm, using non-absorbable material (Serafyl®, 5 metric, USP2; Serag-Wiessner, Naila, Germany). We subsequently connected the transducers with the cervimeter by way of coax cables. The experiments had been approved by the Utrecht University Faculty of Veterinary Medicine committee for the use of animals in research.

*Measurements of Intra-Uterine Pressure changes (IUP)*

After the ultrasound transducers had been attached to the cervix, we introduced the tip of a disposable pipette (Metricure®, Intervet International), with an inner diameter of 3 mm transcervically into the uterine lumen. We inserted a plastic open tip pressure catheter (Corometrics Medical Systems, Wallingford, USA) through the pipette and advanced it as far as possible between the fetal membrane and the uterine wall. After removing the pipette, while carefully fixating the catheter just outside the vaginal opening of the cervix, we fixed the pressure catheter with a single suture at the skin of the vulva. We filled the catheter with

saline solution and connected it to a pressure transducer (Ohmeda Inc., New Jersey, USA), which was fixed to the base of the tail with medical adhesive tape. In one animal (cow no. 2) we were not initially able to insert the pipette properly through the cervix. Only 10 h after the start of measurements, when the caudal cervix had started to dilate, we were able to insert the pipette through the cervix without using force.

*Measurements of changes in cervical dilatation (cervimetry)*

We adapted a cervimeter, as described by Kok *et al*. (1977), for use in cows. Because the distance to be measured in the cow is larger than in humans, we modified the cervimeter to measure a maximal distance of 20 cm instead of 10 cm. To achieve this, we set the frequency of the emitted pulse at 350 KHz, instead of the 600 KHz used in humans. To acquire continuously and display multichannel data (Axotape, version 1.2; Axon Instruments Inc., Foster City, USA), we connected the cervimeter and the pressure transducer to a personal computer through a multichannel analog/digital converter. We used a sampling frequency of 2 Hz to store the data on hard disk, and we started continuous acquisition of data approximately two hours after initiating instrumentation. We left the animals undisturbed, with free access to food and water, as much as possible during the recording. We continuously observed them through a video circuit. Only on a few occasions, we inspected the cow and measuring equipment to assure optimal data acquisition. We removed the transducers again, as soon as the amniotic sac or fetal parts had passed through the vagina and had become visible at the vulva. At that time, the cows showed abdominal straining efforts.

*Data analysis*

*Intra-Uterine Pressure*

Because the pressure catheter was expelled from the uterus on several occasions during the measurements, IUP recordings were not complete and did not allow quantification relative to the moment of PG injection. To study the relation between dilatation and IUP in more detail, we plotted mean values of IUP (mmHg) and dilatation (cm) within consecutive 10 sec periods against time.

*Cervimetry*

When the receiving ultrasound transducer failed to detect an incoming signal, the distance became unrealistically high (100 cm). We discarded such values from the rest of the data. We compressed files by calculating the median values of the distance during consecutive 1 min periods. We plotted the 1 min median values for cervical dilatation against time (h), relative to the moment of PG-injection. We subsequently analyzed these data with a maximum follower procedure. For the maximum dilatation at that time (Figure 1A), we selected every recorded distance that was higher than the previous distance recorded. We connected these points, representing increments in the distance, to calculate a dilatation curve for each cow.

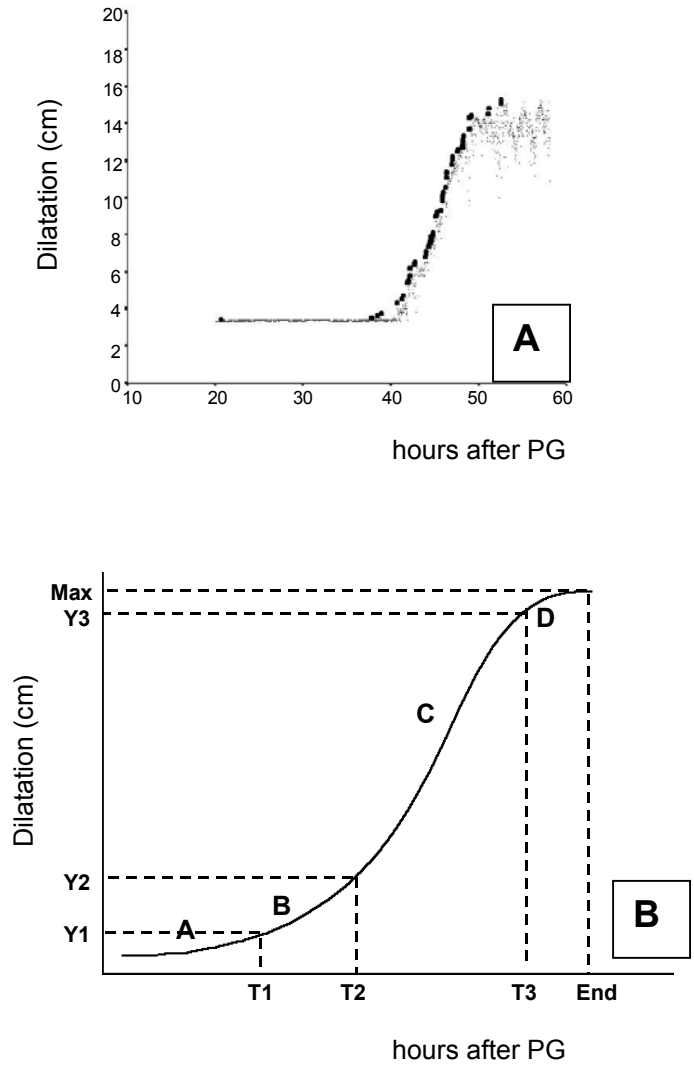


Figure 1. A) Increments in dilatation after maximum follower procedure plotted with time (•) and overlaying a scatter plot (fine points) of 1 minute median values of the dilatation. Example, taken from cow no. 3. B) The slopes of the 4 different phases of cervical dilatation were calculated using a non-linear regression analysis. (A) latent phase, (B) acceleration phase, (C) maximum slope phase, (D) deceleration phase. End is when the transducers were removed and maximal dilatation (Max) is reached (Reproduced after Friedman, 1956).

We further analyzed the dilatation curves to investigate if the different stages of cervical dilatation, as found in women by Friedman (1955; 1956), also exist in cows. As analogy to Friedman (1955; 1956) we divided the dilatation curves into four phases (Figure 1B). During the latent phase of cervical dilatation, there is no appreciable increase in diameter coinciding with a rise in IUP. The second or acceleration phase is marked by a rapid change in the slope of the dilatation curve, until a maximal slope is reached. After this, a phase starts with a linear and rapid dilatation, representing the maximum slope of the dilatation curve. When maximal dilatation is almost been reached, the deceleration phase starts, in which the dilatation slows down considerably, compared to the rate of dilatation during the maximum slope phase. The deceleration phase ends when maximal dilatation has been reached.

*Statistical Analysis*

We performed a stepwise analysis to determine the slopes of the different phases of cervical dilatation in the cow. To this end, we first assessed if the deceleration phase was actually reached in each cow. This deceleration takes place if there is a phase with a significantly smaller slope following the maximum slope phase. Therefore, we analyzed the second part of the dilatation curves [= dilatation > (max. + min.) / 2] by means of a bi-linear non-linear regression model. For this, we used the following regression model:

$$\text{If time} < Tb, \text{predicted value (distance)} = Sa * (\text{time} - Tb) + Yb$$
$$\text{If time} \geq Tb, \text{predicted value (distance)} = Sb * (\text{time} - Tb) + Yb$$

Yb indicates the dilatation at the breakpoint between the two assumed phases. Tb is the time when this point was reached. Sa and Sb are the slopes to be tested. If the 95% confidence interval of both slopes did not overlap, we accepted a statistical significant difference ($P < 0.05$), and if Sb was smaller than Sa, we assumed that the deceleration phase was present.

We performed a non-linear regression analysis to find the slopes of the four different phases in the dilatation curves (Figure 1B). If we registered a deceleration phase, three breakpoints are present. In that case, we used the following regression model:

$$\text{If time} < T1, \text{predicted value} = S1 * (\text{time} - T1) + Y1$$
$$\text{If time} \geq T1 \text{ and time} < T2, \text{predicted value} = Y1 + (\text{time} - T1) * (Y2 - Y1)/(T2 - T1)$$
$$\text{If time} \geq T2 \text{ and time} < T3, \text{predicted value} = Y2 + (\text{time} - T2) * (Y3 - Y2)/(T3 - T2)$$
$$\text{If time} \geq T3, \text{predicted value} = S4 * (\text{time} - T3) + Y3$$

However, if there were only two breakpoints present, we adapted the model as follows:

*If time < T1, predicted value = S1 * (time – T1) + Y1*
*If time ≥ T1 and time < T2, predicted value = Y1 + (time – T1) * (Y2 – Y1)/(T2 – T1)*
*If time ≥ T2, predicted value = S3 * (time – T2) + Y2*

Y is the cervical dilatation in cm at a certain time (T). T1 is the time when the breakpoint between latent phase and acceleration phase was reached. T2 is the time when the breakpoint between acceleration phase and maximum slope phase was reached. T3 is the time when the breakpoint between maximum slope phase and deceleration phase was reached. S1 indicates the slope or dilatation rate during the latent phase in cm/h. The slope of the acceleration phase (S2) is the dilatation rate during that phase. S3 and S4 are the rate of dilatation during the maximum slope phase and the deceleration phase, respectively. S2 and S3 are calculated from the breakpoint values (T, Y), resulting from the applied regression model.

## Results

*Calving Data*

The course of calving varied to such extent (Table 1) that details of the parturient process are given for each animal separately. From the scatter plots (Figure 2A-E), it becomes clear that during cervical dilatation, large variations in diameter occurred. However, there was a temporal increase in diameter until maximal dilatation was reached.

Table 1. Data on the course of calving of the five cows in the experiment

| Cow | Parity | Presentation of the fetus | Course of parturition |
|---|---|---|---|
| 1 | Heifer | Anterior | Vaginal, calf died shortly after birth |
| 2 | Heifer | Anterior | Vaginal |
| 3 | Second | Anterior, oversized fetus | Caesarian[b] |
| 4 | Heifer | Twin pregnancy, first fetus[a] bilateral hip joint flexion, | - |
| | - | second fetus anterior | Vaginal, after repositioning of first fetus |
| 5 | Heifer | posterior | vaginal |

[a] Dead
[b] 40 IU oxytocine im, administered 55 h after PG-injection

Cow no 1.This animal (heifer) delivered the calf in anterior presentation. Acquisition of data started 21 h after PG (Figure 2A). Thirty hours after PG, the unruptured amniotic sac, containing the feet of the calf, protruded from the vulva and we subsequently removed the transducers. Parturition was assisted from that point, and it involved manually stretching (dilatation) of the diaphragm between vagina and vestibulum vaginae. The calf weighed 38 kg but died during delivery. Pathological examinations of the calf revealed aspiration of amniotic fluid.

Cow no 2. We started measurements in this heifer 20 h after PG, and, subsequently, the calf was born in anterior presentation 38 h after PG (Figure 2B). The transducers were removed shortly after the bare feet of the calf had become

visible during abdominal straining. Also, in this case, we had to dilate the diaphragm after which we delivered a 33 kg weighing calf.

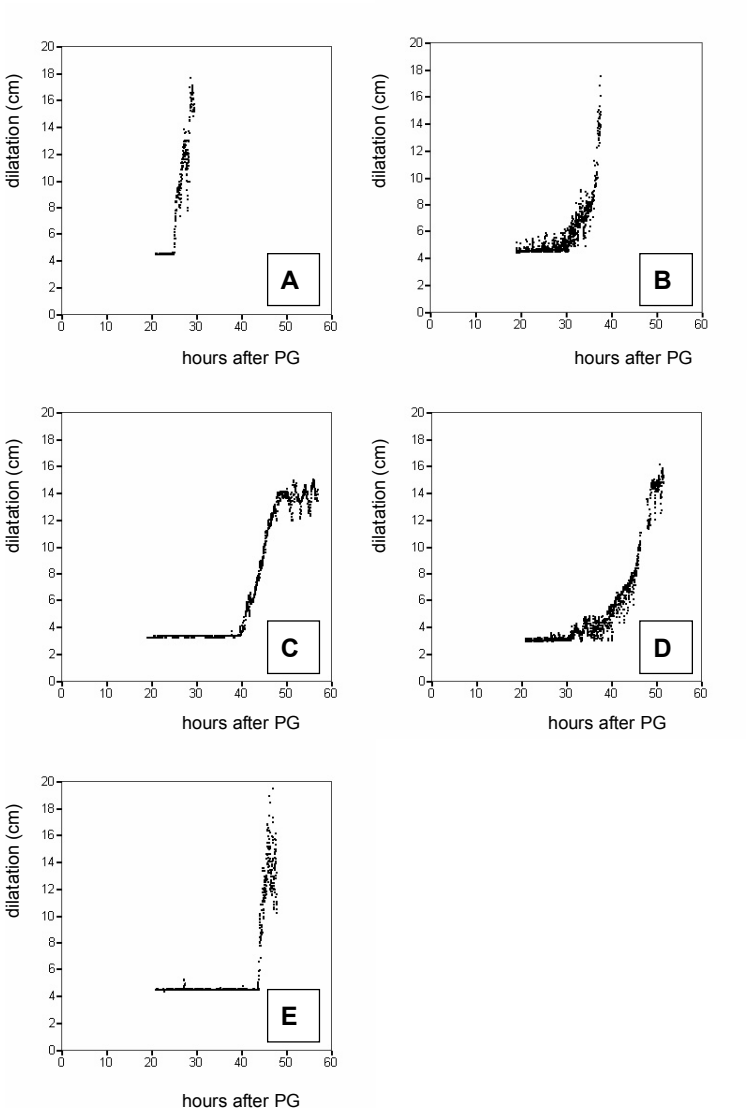


Fig. 2A-E. Scatter plots of the 1 minute median values of dilatation against time for each cow in the experiment.

Cow no 3. We began collecting data 19 h after PG. This second parity cow, showed a plateau phase after maximal dilatation had been reached (Figure 2C). According to the intra-uterine pressure recordings, only contractions with minor amplitude occurred during this stage. Intramuscular administration of 40 IU of oxytocin, 55 h after PG, induced stronger uterine contractions. However, the cervix did not dilate any further, and the cow did not enter the expulsive stage. We removed the transducers 58 h after PG. During an obstetrical examination, we diagnosed an oversized live fetus and subsequently performed a caesarian section. The calf weighed 54 kg.

Cow no 4. At 21 h after PG we began collecting data. Also, this heifer reached a plateau after maximal dilatation (Figure 2D). During this stage we observed large fluctuations in cervical diameter but there was no appreciable gain in dilatation. In spite of abdominal straining efforts of the dam, no fetal parts became visible. Vaginal examination showed the fetus to be in a posterior presentation with bilateral hip-joint flexion. Subsequently, we removed the transducers at 52 h after PG. After repositioning, the already dead fetus (first one of a twin) was born with minimal assistance. The second calf was still vital and was delivered with minimal assistance in anterior presentation.

Cow no 5. We started measurements 21 h after PG. Noticeable in this animal is the long latent period and the very quick rise in diameter (Figure 2E). Two stretched hind legs, in a non-ruptured amniotic sac, appeared at the vulva at about 46 h after PG. After some straining efforts by the cow, the amniotic sac ruptured, abdominal straining immediately ceased and expulsion of the calf did not progress any further. At the same time, the diameter of the cervix had decreased several cm's. At 49 h after PG we removed the transducers. The 40-kg calf was born shortly after that with minimal assistance.

*Changes of cervical diameter in relation to Intra-Uterine Pressure (IUP)*

Although we could not relate the rate of cervical dilatation to uterine activity during the whole process, careful inspection of the recordings, in cases where the quality of IUP was good, revealed several interesting phenomena.

During the latent period, the caudal cervix does not increase in diameter, in spite of apparent increases in the IUP (Figure 3A). However, at an earlier stage in the same cow, we also observed that the diameter of the cervix could increase temporarily in the absence of any measurable increase in IUP (Figure 3B). During the acceleration period and the maximum slope period, the temporary increases in diameter are usually the result of an increase in IUP (Figure 3C), and we notice dynamic changes in diameter. The cervical diameter may fluctuate by increasing or decreasing several cm's; then it may return to its original dilatation or result in a net gain. However, when expulsion of the fetus is stagnated due to fetal pelvic disproportion, such as occurred in cow 3, oxytocin injection augments uterine contractility, but it does not result in further gain in dilatation (Figure 3D).

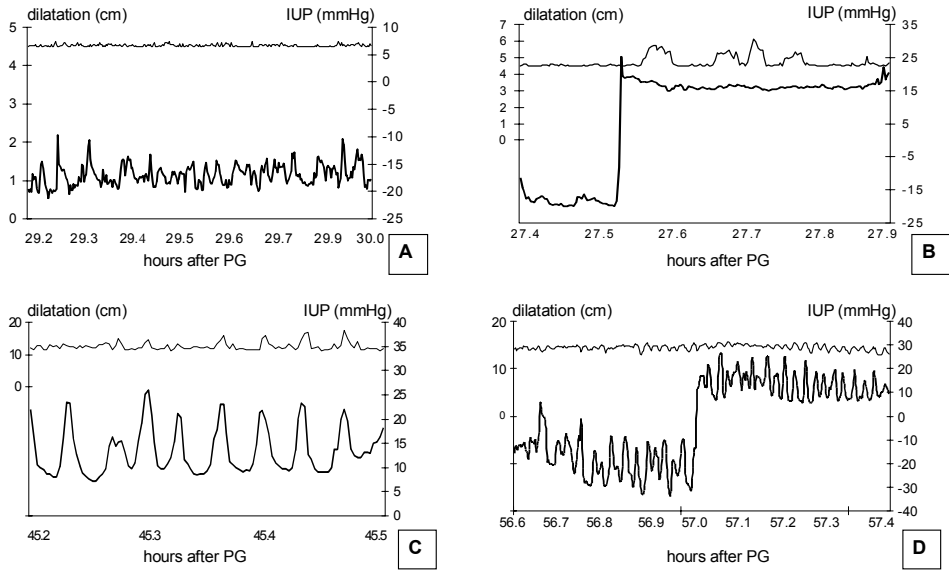


Figure 3. A) During the latent phase, uterine contractions (IUP= bold line) do not seem to influence the dilatation (thin line) of the cervix in the cow. B) However, in that same phase, during moments of uterine quiescence the cervix can increase in diameter, independently from uterine contractions. C) Usually, during the maximum slope phase of cervical dilatation, an increase in IUP is accompanied by an increase in dilatation. D) Forceful uterine contractions do not cause any further gain in cervical dilatation, in the case of an oversized calf. A,B,C originate from the same cow (no. 5). D originates from cow 3. The change in levels of the IUP curves in B and D are caused by a change in position of the cow. The lines represent the mean values of consecutive 10 sec periods.

*Dilatation Curves and Dilatation Rates*

The dilatation curves for each animal are shown in Figure 4. In the dilatation curves of animal 1 and 5, it was not possible to discriminate between an acceleration phase and a phase of maximum slope. In the case of animal 2, we discerned no deceleration phase. The dilatation curves, as derived from the maximum follower procedure, do not show the plateau phase that occurred in cow 3.

In Table 2, we summarize the data obtained through the regression analysis. Because it was not possible to identify the moment at which the parturient pattern of regular uterine contractions began, we calculated the duration of the latent phase relative to the moment of PG injection. Although we registered a deceleration phase in four of the five cases, we were not sure at which time this phase ended in each of those cases, because registrations were ceased at an arbitrary moment. Because of the large variation in calving pattern, we did not

attempt to calculate the mean duration of the different phases of cervical dilatation of the 5 animals.

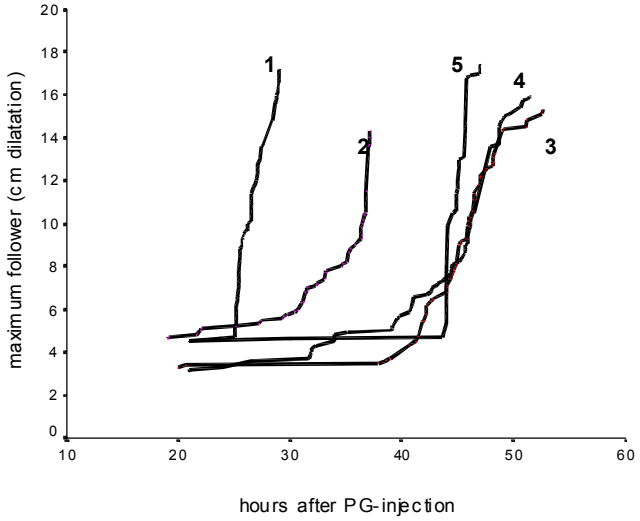


Figure 4. Diagram showing the individual dilatation curves for the five animals after connecting the points from the maximum follower procedure. The numbers denote the number of the animal as mentioned in the text.

Table 2. The slopes or dilatation rates (cm/h) of the different stages of the cervical dilatation curve of the five cows and the duration (hours) of the different phases.

| Cow | Latent period | | Acceleration period | | Period of maximum slope | | Deceleration period | |
|---|---|---|---|---|---|---|---|---|
| | S | Duration[a] | S | Duration | S | Duration | S | Duration[b] |
| 1 | 0.04 | 25.0 | --- | --- | 8.48 | 0.5 | 2.28 | 3.6 |
| 2 | 0.09 | 29.6 | 0.54 | 6.8 | 6.72 | 0.7 | --- | --- |
| 3 | 0.01 | 39.7 | 0.84 | 4.3 | 1.47 | 4.8 | 0.24 | 3.8 |
| 4 | 0.11 | 38.7 | 0.49 | 6.8 | 2.60 | 2.0 | 0.62 | 3.9 |
| 5 | 0.01 | 43.5 | --- | --- | 4.86 | 3.4 | 0.35 | 1.0 |

[a] duration relative to PG,
[b] duration depends on moment of transducer removal

## Discussion

We have demonstrated that it is possible to measure the dilatation of the vaginal cervical opening continuously from early parturition until the start of the expulsive phase, with the use of an ultrasound cervimeter. The measured range of dilatation in the present study was larger than what has previously been reported

for the cow (Gregory, 1977). However, the dilatation curves obtained are in general agreement with the results of Gregory (1977) and Rüsse (1963). At least some of the dilatation curves obtained in this study also appeared similar to the Friedman curve described for women (1955; 1956). However, we should emphasize that the Friedman curve was based on digital examinations and not on continuous recordings. Rüsse (1963) also based his findings on manual examinations, as did Gregory (1977), for the dilatation beyond 7 cm.

The stretched legs of the calf, in the case of anterior presentation, may have influenced the signal detection to such extent that the deceleration phase was not measurable in one of the cows. However, it is also possible that the measurements were simply stopped before the deceleration phase had even been reached. Although it is possible to register the transition of the maximum slope phase into the deceleration phase, it is not possible to calculate the duration of the deceleration phase. In fact, this can only be done when a plateau phase of maximal dilatation is reached. In two of the cows (no. 1 and 5), an acceleration phase could not be determined. This means that the start of the caudal vaginal opening may be rather explosive. The fact that this occurred with a calf in anterior and posterior presentation indicates that the fetal position does not seem to have a large influence.

Several factors, such as fetal weight, type of delivery, fetal position and ripeness of the cervix, influenced the length of the latent phase or the dilatation rate of the maximum slope phase in dilatation curves in women (Friedman, 1955; Friedman 1956). Although the limited number of recordings in our study does not allow us to draw such conclusions for cows, the large variation in the slopes and the form of the dilatation curves obtained in our study suggest that similar factors may be involved in the cow.

At some point after the onset of regular uterine contractions, the cervix will start to dilate in reaction to temporary increases in IUP. In humans, this point has been named the reaction point (Kok *et al*., 1977; van Dessel *et al*., 1991). In our study, it was not possible to identify this point for cows, because IUP recordings were not complete. Electromyographical studies of the bovine uterus, after induction of parturition with PG, show that myometrial activity steadily increases, starting at some 14 h after PG treatment and reaching a maximum after about 26 h (Koets *et al*., 1998). In addition, pressure recordings of the myometrium combined with EMG recordings in spontaneous calving cows show that the frequency and amplitude of pressure increases and electrical bursts directed from uterus to the cervix start to increase 18 to 20 h before expulsion of the calf (Kündig *et al*.,1990). Although we can assume that myometrial activity was already increasing when our measurements started, none of the animals in our study had yet shown any appreciable dilatation of the caudal cervix and had not reached the reaction point. In contrast with our findings, Gregory (1977) reported that uterine contractions only started to occur when the cervix was already passable for one hand. This discrepancy can be explained by the fact that the uterine contractility in Gregory's study was established by means of repeated rectal palpations of the uterus and not by IUP measurements. Although Rüsse (1963), in earlier years, quite elegantly

combined IUP recording with cervical dilatation, the method was restricted because the IUP catheters could only be inserted when the cervix was already passable for one hand. Because of the relatively bad quality of our IUP recordings, we were not able to assess the reaction point for the cervical dilatation, although we succeeded in most cases to insert the IUP catheters at a much earlier stage. By combining the cervimetry with other techniques, such as EMG, that reliably record uterine activity during extended periods of time (Janszen *et al*.,1990), we can expect to gain more insight in the dynamics of cervical dilatation of cows.

During the early latent phase of cervical dilatation, the caudal cervix does not react to a rise in IUP. However, we also observed that the cervical diameter could increase temporarily during the latent phase, when there was no measurable IUP present. Observations that the cervical diameter may even decrease during early labor, synchronously with uterine contractions, have been made in women (Olah *et al*., 1993; Antonucci *et al*., 1997). The authors who made these observations suggested that there is an active musculatory component in the process of cervical dilatation, causing the cervix to contract. Observations in non-pregnant women, that intra-cervical pressure diminishes when uterine contractions are stimulated, have been interpreted as a relaxation of the cervical musculature (Shafik, 1994). As none of these above-mentioned findings were based on the actual recording of muscle activity of the cervix, it is too premature to draw any conclusions about the involvement of a muscle component in the process of cervical dilatation. At a more advanced stage of parturition, mechanical pressure exerted by fetal membranes and fetal body parts, which is progressing into the cervical lumen, is most likely supported by increased IUP during uterine contractions (Figure 3C). In addition, movements of the calf may cause changes in intra-cervical pressure and stimulate cervical dilation, even in the latent phase when the cervix has probably already become softer and may even have started to dilate at the cranial end. Therefore, the observed increase in diameter of the cervix during moments of uterine quiescence may be the result of a momentary relaxation of the circular cervical musculature, perhaps coinciding with increased intra-cervical pressure caused by movements of the calf.

With the technique used in this study, we were able to gain information only about the dilatation of the caudal part of cervix. Real time ultrasonography of the cervix in parturient women has led to the understanding that effacement of the cervix is a dynamic process that begins at the internal cervical os and proceeds in caudal direction (Zilianti *et al*., 1995; Iams, 1997). However, such an approach does not seem suitable for the dilatation of the bovine cervix, because frequently repeated scanning sessions would be needed. This might interfere with the normal physiology of parturition.

The major advantage of the method presented in this study is that it allows continuous measurements without interference in the animals' normal calving behavior. However, we also have to conclude from the present study that in cows, IUP recordings during parturition by means of a transcervical intra-uterine catheter, are not reliable enough to relate the temporal increase of dilatation of the caudal cervix to the degree of uterine contractility.

## Acknowledgements

The authors thank R. van Oord for technical assistance and Dr. H. Jonker for practical advice.

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**-Chapter VI-**

# Cervical dilatation related to uterine EMG activity and endocrinological changes during PGF2α induced parturition in cows

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-submitted for publication-

## Abstract

The temporal relationship between changes in cervical dilatation, uterine electro-myographic (EMG) activity and maternal plasma concentrations of oestradiol 17-$\beta$ ($E_2$), progesterone ($P_4$) and 13, 14-dihydro-15-keto-prostaglandin-$F2_\alpha$ (PGFM) was investigated in parturient cows (n = 6). Calving was induced with as single injection of a synthetic analogue of prostaglandin $F2_\alpha$ (PG) on day 274 of gestation and cervical dilatation and uterine EMG-activity were continuously measured, until the expulsive stage of calving had been reached. In blood samples collected at 4 h intervals, starting at the moment of PG-injection, the mean plasma $E_2$ concentration gradually increased and was significantly elevated at 28 h after PG injection. At 4 h after PG-treatment, the mean $P_4$ concentration had significantly dropped and continued to decrease until a value of around 1 ng/ml was reached, at which level it stayed until the onset of expulsion. Mean plasma PFGM concentrations steadily increased after PG injection, reaching significantly elevated concentrations at 20 h after treatment. In the cows (n = 5), which delivered calves in anterior presentations, Uterine EMG-activity, expressed as root mean square (RMS in $\mu$V), started to increase at a mean interval ($\pm$ SD) of 13.1 $\pm$ 3.7 h following PG- treatment. The increase of EMG-activity was significantly correlated with changes in the plasma PGFM concentrations. In these cows, the dilatation of the caudal cervix started after a mean ($\pm$SD) interval of 28.5 $\pm$ 1.5 h following PG-treatment and dilatation progressed at mean ($\pm$ SD) rate of 2.25 $\pm$ 0.24 cm/h. In one cow, with a calf in posterior presentation, uterine EMG-activity and dilatation started at 15.8 h and 31.8 h respectively after induction of calving. It is concluded that a well predictable sequence of physiological changes occurs around induction of calving, which allows specific timing of future studies on cellular and biochemical changes within the cervix during parturition.

## Introduction

Our understanding of the mechanisms involved in cervical ripening and dilatation has been hampered by the lack of studies in which the onset of labour has been well defined with respect to simultaneous changes in uterine activity and plasma hormone concentrations. Most clinical studies on the relation between uterine activity and cervical dilatation in women were possibly flawed, because they depended on an arbitrary starting point (Friedman, 1955; Friedman 1956; van Dessel *et al*., 1994; Antonucci *et al*., 1997) i.e. the time of admittance, and/ or patient recall of the start of uterine contractions. Consequently, the latent phase of cervical dilatation, that is the stage of parturition in which there is no appreciable dilatation, could not be accurately defined in these women. As a result, the acceleration point, at which dilatation starts to speed up (Friedman, 1955; Kok, 1977; Friedman 1956; van Dessel *et al*., 1994), could not be accurately determined. In one report, serial blood sampling was performed in an attempt to describe the temporal relationship between cervical dilatation, uterine activity and hormonal changes in women (Fernandes *et al*., 1995). Unfortunately, this report may have been biased because it was based on digital, and thereby subjective, measurements of the cervical diameter, that had been repeated at various intervals. It has been claimed that the use of digital measurements may cause artefacts in the partograms of women, leading to falsely assumed phases during cervical dilatation (van Dessel *et al*., 1994: Impey *et al*., 2000), including the deceleration phase proposed for women by Friedman (1955, 1956). Except for women (Kok, 1977; van Dessel *et al*., 1994; Olah *et al*., 1994; Antonucci *et al*., 1997), attempts to combine measurements of uterine activity with cervical dilatation have only been made in the cow (Rüsse, 1963, Gregory, 1977, Breeveld-Dwarkasing *et al*., 2002). Similar to the studies in women, the two studies in cows on spontaneous calvings (Rüsse, 1963, Gregory, 1977) did not adequately define the beginning of parturition.

Dilatation of the cervix depends on the resistance caused by the visco-elastic properties of the cervix on the one hand and the force induced by uterine contractions on the other hand. Softening of the cervix is an important prerequisite for cervical dilatation. Recent studies in rats suggest that both cervical resistance and collagen concentration within the cervix already start to diminish slowly by the end of the second trimester and reach a nadir at two-thirds of the third trimester (Shi *et al*., 1999). During labour additional structural changes may take place before the onset of dilatation. It has been shown that cervical dilatation is more closely related to the concentration of collagenolytic enzymes in cervical tissue than to the duration of labour per se (Winkler *et al*., 1999). This implies that local changes in the cervix, unrelated to uterine activity may play a role in dilatation during labour (Winkler *et al*., 1999). In fact, cervical effacement in parturient sheep took place independently from uterine contractility or pressure, when the uterus was surgically separated from the cervix (Ledger *et al*., 1985) or when uterine activity was suppressed with $\beta_2$-mimetics (Owiny *et al*., 1992). Nonetheless,

cervical dilatation does not occur without uterine activity. According to Lindgren (1973), the speed of dilatation depends on the frequency and strength of the uterine contractions, while the head-to-cervix pressure may serve as an expression of the tissue resistance of the cervix against the intra-amniotic fluid pressure changes, caused by uterine contractions. In addition, van Dessel *et al*., (1994) claimed that more contraction work (= the sum of all active pressure areas over 1 cm of cervical dilatation) is needed for cervical dilatation, before than after the acceleration point, which implies that even during parturition changes take place in the cervix, which influence its resistance against uterine contractions. The observation that the relationship between intra uterine pressure and head-to-cervix force varied widely between women, while a close correlation between the two variables did not consistently result in faster dilatation (Allman, 1996), indicates that the relationship between the uterine contractile force and cervical dilatation is not a simple one. In ruminants where the pregnant uterus does not rest upon the pelvic floor, mechanics of cervical dilatation can expected to be different from that in women.

Over the years, several instruments have been designed to measure cervical dilatation continuously (van Dessel *et al*., 1991; Lucidi *et al*., 2000), but they have not been used in combination with EMG recordings of uterine activity and serial blood sampling, probably because of ethical (women) and anatomical (small laboratory animals) restrictions in the species that were under study. The objective of the present study was to design an experimental model that would allow us to investigate the temporal relationship between changes in cervical dilatation, uterine EMG activity and endocrinological changes in the cow. To this end, a previously described method of ultrasound cervimetry [9] was combined with recordings of uterine EMG activity and measurements of maternal plasma hormone concentrations, in term pregnant cows during $PGF_{2\alpha}$ (PG) induced parturition at term.

## Materials and Methods

*Animals and Surgery*

Six multiparous Holstein Friesian cows with singleton pregnancies were used in this experiment. During the experimental period the cows were housed in individual stands and subjected to a normal daylight cycle. Food and water were given ad lib according to the feeding standards of dry cows. The protocol was approved by the Animal Research Committee of the Faculty of Veterinary Medicine of Utrecht University. After a 48 h fast and 24 h of water deprivation, surgery was performed under general anaesthesia at least ten days before the induction of parturition, as previously described (Janszen *et al*., 1990). In short, with the cow in a dorsal recumbent position, a mid ventral laparotomy was performed during which two bipolar silver electrodes were sutured, approximately 30 cm apart, on the pregnant uterine horn. Subsequently, the cow was turned to the right lateral position for catheterisation of the left circumflex artery. The wires and catheter

were tunnelled subcutaneously to the dorsal area of the sub-lumbar fossa, wrapped in alcohol soaked gauze pads and kept in a plastic bag until experimentation. Ampiciline (12 mg/kg bodyweight; Praxavet Ampi-15, Boehringer Ingelheim, Alkmaar, The Netherlands) was given i.m. once a day during the first 5 days following surgery.

*Experimental protocol*

Starting at day 270 of gestation, blood samples were collected daily between 8.00 and 10.00 a.m in heparinized tubes for later analysis of maternal plasma concentrations of progesterone ($P_4$) and oestradiol-17$\beta$ ($E_2$). On day 274 of gestation, two ultrasound transducers, one transmitter and a receiver, were sutured on the vaginal cervical rim as previously described in detail (Breeveld-Dwarkasing *et al*., 2002). The change in transit time of the ultrasound signal was used as to measure the distance between the transducers continuously. Calibration in water was used to check linearity of the recorded distance and the distance between the transducers.

Simultaneous recordings of cervical dilatation and uterine EMG activity were started between 12.00 and 14.00 p.m., shortly after the ultrasound transducers had been inserted and the cow had received 7.5 mg Luprostiol i.m. (Prosolvin, Intervet, Boxmeer, The Netherlands). A cervimeter as described earlier (Breeveld-Dwarkasing *et al*., 2002) was used to measure cervical dilatation. A universal amplifier (model 13-4615-56, Gould Inc. Cleveland Ohio) was used to measure uterine EMG activity; the band pass filter was set between 1 and 10 Hz. A sampling rate of 40 Hz was used to continuously acquire and display the multi-channel data (Labview 5.0, National Instruments, Dublin, Ireland) and the digitalized signals were stored on PC. From the moment of PG-treatment (T0) onwards, arterial blood samples were collected every 4 hours through an extended cathether and in heparinized tubes for later analysis of maternal plasma concentrations of $P_4$, $E_2$ and 15-ketodihydro-$PGF_{2\alpha}$ (PGFM). During the measurements, the cows were left undisturbed in an individual pen with free access to food and water. Recording took place in an adjacent room, while the cow was observed by television camera. At the onset of the expulsive stage, when the amniotic sac had become visible outside the vulva, recording was discontinued and the ultrasound transducers were removed from the dilated cervix to prevent damage during the expulsion of the calf.

*Hormone analysis*

$P_4$ concentrations were measured by a validated direct solid phase $^{125}I$ radioimmunoassay (RIA) as previously described (Dieleman and Bevers, 1987). The sensitivity of the assay was 47 pg/ml; the inter-assay coefficient of variation was 11% (n = 16) and the intra-assay coefficient of variation was 7.5% (n = 20). $E_2$ concentrations were measured by RIA as previously described (Dieleman and Schoenmakers, 1979) following double diethylether extraction. The sensitivity of the assay was 32 pg/ml and the inter-assay and the intra-assay coefficient of variation were both 9% (n = 20). PGFM concentrations were measured by

homologous double antibody RIA as previously described (Granström and Kindahl, 1982). The sensitivity of the method was 30 pmol/L, the inter-assay coefficient of variation was 14% and the intra-assay coefficient of variation ranged between 6.6% and 11.7% for the different ranges of the standard curve.

*Data analysis and Statistical analysis*
*Maternal oestradiol-17β, progesterone and $E_2$ / $P_4$ ratio*

The data were analysed with INSTAT (1990, Graph Pad Software, San Diego, California, USA) and are presented as means ± SEM. The samples that were obtained in the morning of day 274 were designated as control values prior to PG-treatment. The plasma hormone concentrations and $E_2$ / $P_4$ ratios were analysed for the effect of time both before and after treatment, using a repeated measures ANOVA (RM-ANOVA), assuming a Gausian distribution. If a significant effect of time was present, post hoc analysis was performed by comparing mean values to pre-treatment controls, using Dunnetts test. A p value < 0.05 was taken as the level of significance.

*Cervical dilatation*

The data were analysed with SPSS (1997, Chicago, Illinois, USA). For each cow, the mean dilatation (cm) within each hour following the PG treatment was calculated and the values were plotted relative to T0. Because we were interested primarily in the onset and speed of cervical dilatation, we used non-linear regression (NLR) analysis to identify the point, of transition from the latent phase with no appreciable increase in cervical diameter, into the dilatation phase. The following model was used:

*If time < T1; predicted value (of dilatation at a given time during latent phase)*
*= S1 × (time − T1) + Y1*

*If time ≥ T1; predicted value (of dilatation at a given time during dilatation phase)*
*= S2 × (time − T1) + Y1*

T1 represents the breaking point in hours after T0 (PG treatment). Y1 is the average cervical dilatation (in cm) during the latent phase and S1 is the rate of dilatation (cm/h) during the latent phase. S2 is the rate of dilatation during the dilatation phase. It was assumed that cervical dilatation progressed in a linear fashion until maximum dilatation was reached; the deceleration phase, if present, was not included in the calculation.

From the results obtained for the individual cows, the overall mean interval (± SD) from PG treatment to the onset of cervical dilatation was calculated, as well as the overall, mean (± SD) dilatation rate. A mean dilatation curve from the values obtained for the individual cows, was plotted as the mean dilatation per hour (± SEM) relative to T0. A maximum follower procedure as previously described (Breeveld-Dwarkasing *et al*., 2002) was used to find the absolute maximum

diameter of the cervix that was reached, before the transducers had been removed from the cervix.

*Uterine EMG activity in relation to cervical dilatation and PGFM concentrations*

For each of the two EMG electrodes mean, root mean square (RMS) values were calculated for each hour after PG injection. Because the onset of electrical uterine activity was unrelated to the position of the electrode, data from the 2 electrodes were pooled in the case of cows # 1, 2, 4 and 5, before they were further analysed together with data of the remaining two cows in which only one electrode functioned properly. The NLR model described above, was also used to find the transition point between the phase of almost complete uterine quiescence, that is associated with the PG induced luteolysis (Janszen *et al*., 1993) and the onset of uterine activity:

$$\text{If time} < T1_e\text{; predicted value (of EMG activity at any given time during quiescence)} = S1_e \times (\text{time} - T1_e) + Y1_e$$

$$\text{If time} \geq T1_e\text{; predicted value (of EMG activity at any given time during increasing activity)} = S2_e \times (\text{time} - T1_e) + Y1_e$$

$T1_e$ is the transition point for electrical activity, in hours after T0. $Y1_e$ is the mean RMS value ($\mu$V) and $S1_e$ is the electrical activity rate (slope, $\mu$V/h) during the phase of uterine quiescence. $S2_e$ is the electrical activity rate during the phase of uterine activity. The first 4 h of the EMG recordings were not included in the analysis because immediately after PG injection a period of 1-2 hours with increased uterine activity ussually occurs (Janszen *et al*., 1993; Koets *et al*., 1998). The interval between the transition points of uterine electrical activity and cervical dilatation was calculated for each individual cow, from which the overall mean ($\pm$ SD) was calculated. A mean curve for electrical activity was constructed from the values obtained for the individual cows, by plotting the mean RMS values per hour ($\pm$ SEM) relative to T0.

Mean PGFM concentrations were also plotted and analysed for an effect of time with a RM-ANOVA, followed by Dunnetts to compare mean values against T0 as control value. Pearsons correlation test was used to study the relation between uterine electrical activity, cervical dilatation and PGFM concentrations. A p value < 0.05 was taken as the level of significance.

## Results

*Outcome of Calvings*

All six cows had normal vaginal deliveries. Five cows delivered their calves in an anterior presentation, between 35.3 h and 41.2 h after PG treatment (mean: 37.7 ± 2.5 h after PG), while one cow delivered a calf in a posterior presentation,

52.9 h after the injection. Recordings were discontinued at the onset of the expulsive stage, between 33.6 h and 40.4 h after PG in the five cows with anterior presentations (mean: 36.9 ± 2.8 h after PG) and 51.9 h after PG in the cow with the posterior presentation.

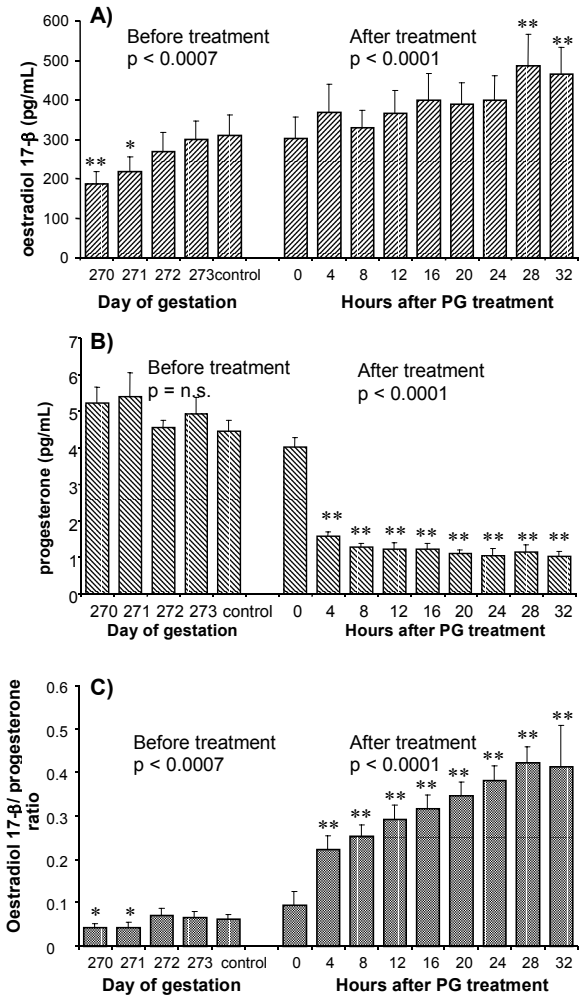


Figure 1. Mean (± sem) maternal plasma concentrations of oestradiol -17β (**A**), progesterone (**B**) and the oestradiol17-β/ progesterone ratio (**C**), before and after injection of 7.5 mg Luprostiol, a synthetic $PGF_{2\alpha}$.on day 274 (control). The significance of time effect (RM-ANOVA) in the before and after treatment period is indicated in the figures, n.s. means non-significant. The asterisks indicate significant differences compared to the before treatment control at day 274 (Dunnets test, * $p < 0.05$, ** $p < 0.01$).

*Maternal oestradiol-17β, progesterone and $E_2$ / $P_4$ ratio*

Mean hormone concentrations were calculated only up until 32 h after PG treatment, because from the next sampling moment (36 h after PG) on, one or more of the cows had calved already.

Plasma $E_2$ concentrations before PG-treatment showed a significant effect of time ($p < 0.0007$). At day 270 and 271 (186 ± 31 pg/ ml and 217 ± 38 pg/ ml, respectively) they were significantly lower ($p < 0.01$ and $p < 0.05$ respectively) than at day 274 (308 ± 53 pg/ ml). After PG treatment, $E_2$ concentrations increased further with a significant time effect ($p < 0.001$) and were higher than the control ($p < 0.01$) from 28 h (486 ± 80 pg/ ml) onwards (Fig. 1A). The $P_4$ concentrations gradually decreased in the period before PG-treatment from 5.7 ± 0.4 ng/ ml to 4.9 ± 0.5 ng/ ml, but there was no significant effect of time. A significant effect of time on the $P_4$ concentrations was present in the period after PG-treatment ($p < 0.0001$). Within the first 4 h after PG treatment, $P_4$ concentrations had dropped significantly (1.6 ± 0.1 ng/ ml, $p < 0.01$) compared to the control on day 274 and thereafter, they slowly and gradually decreased, until plateau values of around 1 ng/ ml were reached (Fig. 1B). With the ratios being significantly lower at days 270 and 271 (0.04 ± 0.01 and 0.04 ± 0.02, respectively, $p < 0.05$) than the control values (0.06 ± 0.01), there was a significant effect of time on the $E_2$/ $P_4$ ratio ($p < 0.0007$) in the period before PG-treatment. There was also a significant time effect in the period after PG-treatment ($p < 0.0001$) and from 4h (0.22 ± 0.03) until 32h (0.41 ± 0.09) after the PG injection, the $E_2$/ $P_4$ ratios were significantly higher than the control value at day 274 ($p < 0.01$, Fig. 1C).

*Cervical dilatation*

The presentation of the calf clearly influenced the characteristics of the dilatation curves (Fig. 2). Therefore, the cow with posterior presentation was excluded from the calculation of the mean cervimetry data. For reasons of comparison however, the data obtained from this single cow will be mentioned in the text. The maximal diameter of the caudal cervix, that had ever been reached before the transducers were removed from the caudal cervix, varied between 11.4 cm and 20.2 cm in the cows with the anterior presentations and was 15.5 cm in the cow with the posterior presentation. Fig 2 shows the dilatation curves based on the mean dilatation per hour for each cow. The point, at which the dilatation phase started, was calculated at 28.5 ± 1.5 h after PG injection. After this point, the dilatation increased almost linearly at a rate of 2.25 ± 0.24 cm/h. The time interval between the onset of dilatation and expulsion of the calf was 9.3 ± 2.1 h. In the case of the cow with a calf in posterior presentation, dilatation started at 31.8 h with a dilatation rate of 1.57 cm/h, while a plateau was reached at 40.9 h and the calf was born 12.1 h later. The data for the individual cows are shown in Table 1.

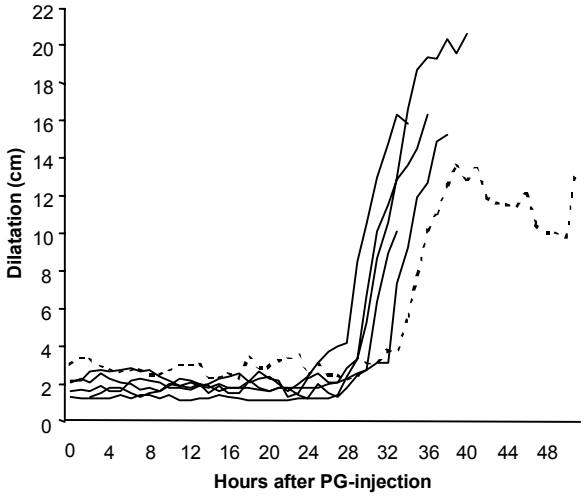


Figure 2. Dilatation curves: mean dilatation per hour as a function of time (in hours) after the PG injection. The straight lines represent the cows with a calf in anterior presentation. The broken line represents the dilatation curve of the cow that delivered a calf in a posterior presentation.

Table 1. Onset of increase in uterine activity and cervical dilatation (hours after PG injection) and the dilatation rates (cm/hour).

| Cow | Onset uterine activity (h) | Onset dilatation (h) | Onset uterine activity – onset dilatation (h) | Dilatation rate (cm/h) |
|---|---|---|---|---|
| # 1 | 13.5 | 28.8 | 15.3 | 2.15 |
| # 2 | 10.3 | 28.7 | 18.4 | 2.60 |
| # 3 | 13.0 | 26.5 | 13.5 | 2.25 |
| # 4 | 19.0 | 30.6 | 11.6 | 1.94 |
| # 5 | 9.8 | 27.6 | 17.8 | 2.29 |
| Mean (SD) | 13.1 (3.7) | 28.5 (1.5) | 15.3 (2.7) | 2.25 (0.24) |
| # 6 | 15.8 | 31.8 | 16 | 1.57 |

Cows #1-5 with calf in anterior presentation, cow # 6 with calf in posterior presentation.

*Uterine EMG activity in relation to Cervical dilatation and PGFM levels*

Five of the 6 cows showed a period of increased uterine activity immediately after the PG treatment that lasted ≤ 4 hours. Thereafter, all the cows showed a period of almost complete myometrial quiescence, during which the caudal cervical diameter could temporarily increase for approximately 1- 1.5 cm independent of uterine activity (Fig 3A). During the latent phase of cervical dilatation, uterine activity increased gradually and some hours later, the cervical

diameter began to respond to the uterine contractions (Fig 3B). During the dilatation phase, uterine activity increased even more, while the cervical reactions became more synchronised, showing larger excursions of the diameter during the uterine contractions (Fig 3C).

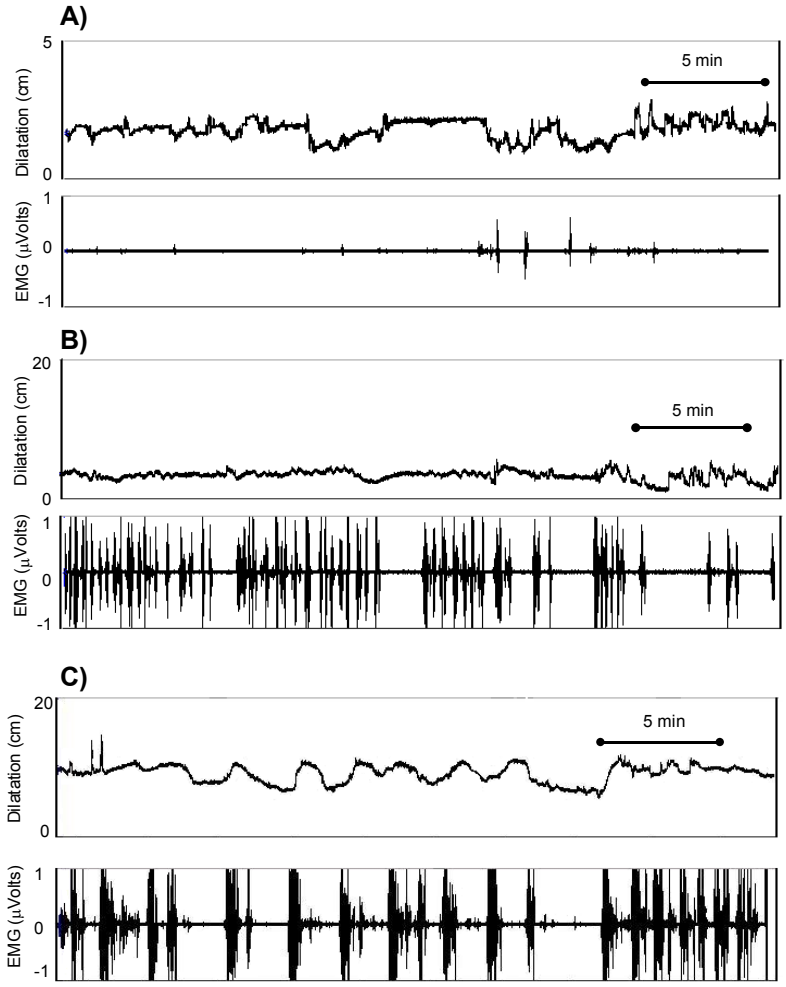


Figure 3. Examples of synchronous recording of cervical dilatation and EMG. **A**) Almost complete myometrial quiescence, during the early latent phase. Small excursions of the cervical diameter may occur, unrelated to uterine activity. **B**) Beginning of dilatation phase: high intensity, short-term electrical bursts, occurring frequently on which the cervix reacts. **C**) Dilatation phase: increasing intensity of electrical bursts on which the cervical diameter reacts more synchronously and with larger excursions. Please notice the difference in scale between the vertical axis of **A**) compared to **B**) and **C**).

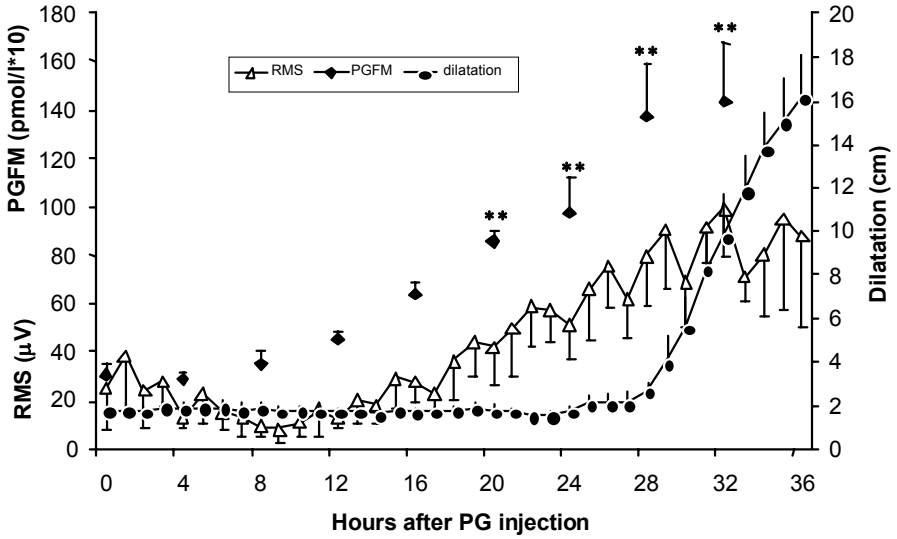


Figure 4. Plasma 15-keto-dihydro-$PGF_{2\alpha}$ concentrations (n = 6), as a function of time (in hours) after prostaglandin injection, related to myometrial activity expressed as root mean square values (RMS, n = 5) and, to cervical dilatation in cm/ h (n = 5). Values represent means ± SEM. Mean PGFM concentrations with asterisks are significantly higher than at 0 h after PG injection (** $p < 0.01$).

Uterine activity expressed as RMS values started to increase 13.1 ± 3.7 h after PG. In the cow with a calf in posterior presentation, RMS values started to increase at 15.8 h after PG (Table 1). The mean interval between the onset of uterine activity and the onset of cervical dilatation was 15.3 h ± 2.7 h. Figure 4 illustrates the relation between mean PGFM concentrations, mean uterine activity per hour and mean cervical dilatation per hour, for the cows with a calf in anterior presentation. Plasma PGFM concentrations started to increase gradually from 8 h after PG treatment onwards. There was a significant time effect ($p < 0.0001$) and at 20 h after PG treatment onward they were significantly higher (860 ± 46 pmol/ L, $p < 0.01$) than at T0 (305 ± 46 pmol/ L, Fig. 4). The PGFM concentrations also showed a significant correlation to uterine EMG activity from T0 until 32 h after PG treatment ($R = 0.97$, $p < 0.01$). However, no relation was found between the rate of increase in uterine activity and the rate of dilatation (data not shown).

## Discussion

The results from this study demonstrate that in prostaglandin induced calvings, a well-defined sequence of events takes place with respect to changes in the maternal plasma concentrations of $E_2$, $P_4$ and PGFM, the activation of the myometrium and the dilatation of the caudal cervix. The interval between the increase in myometrial activity and the expulsion of the calves in this experiment is in agreement with published data for spontaneous (Kundig *et al*., 1990) and PG induced calvings (Koets *et al*., 1998). Like has been reported for spontaneous calving cows (Pope *et al*., 1969; Stabenfeldt *et al*., 1970; Edqvist *et al*., 1973; Hunter *et al*., 1977; Möstl *et al*., 1981), we found a progressive increase in plasma $E_2$ concentrations and a progressive reduction in $P_4$ concentrations towards term. The significantly increased $E_2$/ $P_4$ ratio at day 274, compared to day 270, and additional significant increase of this ratio shortly after the PG injection, is also in agreement with the findings of Fuchs *et al*., (1992) for the plasma oestrogen/ progesterone ratio of term pregnant and spontaneous calving cows. Therefore, we conclude that the cows used in the present study were very close to term at the time that PG was injected to induce luteolysis. The rise in $E_2$ concentrations that already started a few days before the induction of parturition, may have played a role in the formation of gap-junctions in the myometrium (Cole and Garfield, 1986) and in this way may have prepared the myometrium for co-ordinated uterine activity. It may also have stimulated the synthesis of $PGE_2$ receptors in the cervix during the last few days of pregnancy as has been reported for the baboon (Smith *et al*., 2001), thus sensitising the cervix to $PGE_2$. $PGE_2$ may have stimulated the final functional changes in the cervix, including increased water content and changes in the content or composition of proteoglycans, shortly before or even during parturition, as has been found in several other species, including ruminants (Winkler and Rath, 1999; Chwalisz and Garfield, 1998; Fossang *et al*., 1984; Cabrol *et al*., 1981; Leppert *et al*., 2000).

The classical description of cervical dilatation for women by Friedman (1955; 1956), includes a latent phase, an acceleration phase, a phase of maximum slope and a deceleration phase and is often referred to in studies of cervical dilatation. In a previous study (Breeveld-Dwarkasing *et al*., 2002), we demonstrated that an acceleration phase and a deceleration phase are not always present during cervical dilatation in the cow. If figure 2, where the mean dilatation per hour has been plotted, it can be seen, however, that dilatation in all the cows started gradually before it progressed into the rapid dilatation phase, equivalent to an acceleration phase. Obviously, the outcome of cervimetry experiments are influenced by the method of analysis as illustrated by the fact that when the present data were analysed, using a maximum follower procedure followed by a search for transition points as we did previously (Breeveld-Dwarkasing *et al*., 2002), we found an acceleration phase in only two and a deceleration phase in only four of the six cases. Because, our main interest was to find the onset and rate of cervical dilatation, we choose to assess the point of intersection of the slope of the latent phase (S1) and the slope of the dilatation phase (S2) with a two-compartment

model, assuming linear progression of cervical dilatation in both compartments. With the use of this model, we observed only a small variation in transition points of cervical dilatation and in dilatation rates in the 5 cows with anterior presentation (Table 1).

The results of this study, demonstrate that differences in the presentation of foetal parts between an anterior and posterior presentation of the calf, has implications for the progress of parturition and for the mechanical part of cervical dilatation. This clearly influences the forms of the dilatation curves and which is also in line with our earlier observations (Breeveld-Dwarkasing *et al*., 2002). In addition, the large variation in the maximal diameter that was found between animals that calved with anterior presentations, may also be explained from physiological variations in, which of the foetal parts have already entered the cervical canal and, in the degree to which they have entered it, when the expulsive stage begins.

The temporary increase of myometrial activity shortly after PG injection as observed in 5 of the 6 cows has been reported before (Janszen *et al*., 1993; Koets *et al*., 1998). This increase in activity is probably due to a direct effect of the synthetic $PGF_{2\alpha}$ on the myometrium, and not by uterine $PGF_{2\alpha}$ production as PGFM levels were not significantly elevated at that time. Plasma PGFM concentrations however, may not only reflect the $PGF_{2\alpha}$ turnover. The enzyme 9-keto-prostaglandin E(2) reductase, which has been shown to convert $PGE_2$ to $PGF_{2\alpha}$ is present in the bovine endometrium (Asselin and Fortier, 2000) and for rats it has been suggested that progesterone withdrawal during oestrogen exposure stimulates the production of the enzyme (Peplow, 1991). Thus, it is possible that an increased production of $PGE_2$ in the uterus and cervix, after conversion to $PGF_{2\alpha}$ has also contributed to the rise in maternal plasma PGFM concentrations.

We did not find a relation between the rate of increase in uterine activity and the rate of dilatation of the caudal cervix, which is in accordance with observations made by Almann *et al*., (1996) in women. The absence of a linear relationship does not mean that uterine contractions are unnecessary for cervical dilatation. Rather, it suggests that the timing of the final structural changes in the cervical tissue during parturition play a decisive role in the response of the cervix to uterine contractions. It has been postulated that the period of myometrial quiescence associated with prepartum luteolysis in the cow is an important phase for biochemical and morphological preparations of the cervix and myometrium for labour (Janszen *et al*., 1990; Janszen *et al*., 1993). The duration of the time interval between the onset of uterine activity and dilatation of the caudal cervix as found in this study, allows the cervix to undergo further structural changes while uterine activity increases, in order to prepare for the phase in which it can give in to the forces induced by the contractions. Taking cervical biopsies during this time should reveal what these changes are and which factors are involved.

Nitric oxide (NO) might be one of the candidates that play a role in the cervix during parturition. In rats, the concentrations of nitric oxide synthase (NOS)

and its mRNA specifically increase in the cervix, between the initiation of parturition and the delivery of the first pup (Buhimishi *et al*., 1996; Ali *et al*., 1997). NO may induce connective tissue changes, through the stimulation of glycosaminoglycan synthesis, metalloproteinases and $PGE_2$ synthesis via cyclo–oxygenase II (Garfield *et al*., 1998; Chwalisz and Garfield, 1998; Winkler *et al*., 1999; Winkler and Rath, 1999), and these processes may take place especially during the latent phase of cervical dilatation. In addition to its role in the regulation of structural changes in the connective tissue, it might be hypothesised that NO also induces relaxation of the cervical muscles during parturition such as might occur in the myometrial muscles during gestation. EMG activity has been observed in the cervix of several species during parturition (Toutain *et al*.,1983; Oláh *et al*., 1993; Panjtar and Verdenik, 1995) and combined measurements of cervical EMG and mechanical activity (by way of strain gauges) of the cervix indicate that an active muscle component is involved in the functional changes of the cervix during parturition in sheep (Toutain *et al*.,1983). The observations that cervical EMG activity in unripe cervices is significantly greater than in riper cervices of women in labour (Panjtar and Verdenik, 1995) and that electrical activity in the cervix reduces during softening (Oláh, 1994), suggest progressive muscle relaxation during the final ripening stage of the cervix, in which NO may have a regulatory role.

Besides EMG-activity, there are other indications that during the early stage of cervical dilatation cervical muscle is active. The temporary increases in cervical diameter in the absence of uterine electrical activity during the latent phase as observed in the present and previous study (Breeveld-Dwarkasing *et al*., 2002) may be postulated to reflect contractions longitudinal muscles in the cervix that pull the vaginal cervical rim backwards and open. The reductions in cervical dilatation synchronously with uterine contractions as reported during the early stages of dilatation in women (Oláh *et al*., 1992; Antonucci *et al*., 1997), may than reflect contractions of the more circular oriented muscles of the cervix. These observations made by us and by others indicate that an active muscle component is present in the cervix during parturition, which may also be influenced by NO.

Our study has demonstrated that three different episodes can be recognised in the temporal relationship between cervical dilatation and uterine EMG activity in parturient cows: 1) the period between luteolysis and the onset of uterine activity 2) between the onset of uterine activity and the onset of cervical dilatation and 3) the actual dilatation phase. This model provides a basis for future studies, on structural changes in the cervix relative to the stage of labour, as defined by objective physiological and endocrinological landmarks of parturition.

## Acknowledgements

We thank, Dr. Á. CS. Bajcsy (Department of Obstetrics and Reproduction, University of Veterinary Science, Budapest, Hungary), Ms. R. van Oord (Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University) and Drs. R. de Jong, for their assistance during the preparations of the

experiment and the data collection. We also thank Dr. S.J. Dieleman and Ms. T. Blankenstein (Department of Farm animal Health) for performing the $E_2$ and $P_4$ assays and Dr. E. Mulder (Wilhelmina Children Hospital, Utrecht, the Netherlands) for help with the statistical analysis of the hormonal data.

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**-Chapter VII-**

# Summarising Discussion

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## Cervical softening during the oestrous phase of the cycle

Softening of the bovine cervix during oestrus has already been described in earlier years (Hammond, 1927; Herrick, 1951), but it is also well known by veterinary clinicians and AI-technicians that during oestrus the cervix feels softer to the touch and also is easier to penetrate with an insemination pipette. Differences in texture of the cervix between the oestrous phase and the rest of the cycle have also been reported for other species (dog: Silva *et al*., 1995; horse; Day *et al*., 1995; rats: Hamilton, 1947; pigs: Rigby, 1967), although the results are not always uniform. For example, for the pig it was reported that increased oedema of the cervix explained the increased rigidity of the cervix that was found in pigs slaughtered during oestrus (Rigby, 1967), whereas, in the others pecies, the reported changes pointed to a softening and/or opening of the vaginal part of the cervix during oestrous. No doubt, the reported differences between species reflect the differences in type of copulation and the place of subsequent semen deposition during natural mating. They may also be associated with the difference in timing between physical signs of oestrous and the moment of ovulation. Both these factors could determine how the spermatozoa should be transported or may be even stored for some time within the cervix. In that case the anatomical structure and the amount of structural changes in the cervix may have a regulatory role. For example, in the oestrous sheep it has been described that the cervix actively opens and contracts, literally drawingthe semen from the vagina into the cervix after semen was deposited in the vagina by means of artificial insemination (King *et al*., 1998).

In addition to the reports of a macroscopically softened cervix during oestrous, histological evidence of increased leucocyte invasion, which can be associated with connective tissue changes, has been reported for the cervix of humans (Kemp *et al*., 1998) and rats (Hamilton, 1947). Several factors that can be associated with the induction of softening in the non-pregnant cervix, are reported to have changed during oestrous, such as the increased expression of IL-8 genes in sheep (Mitchell *et al*., 1998) and of oxytocin receptors in sheep (Matthews and Ayad, 1994) or cows (Fuchs *et al*., 1996). Oxytocin in turn, might stimulate the production of cyclo-oxygenase and $PGE_2$ in cervical tissues of oestrous cows, which is inhibited by progesterone (Shemesh *et al*., 1997). In general, it is believed that oestrogen up-regulates the oxytocin receptor in the cervix and at least for the rat (Umscheid *et al*., 1998) this was proven to be the case. The presence of oestrogen and progesterone receptors in the cervix, also indicates that these steroid hormones are involved in the regulation of functional processes in the cervix during the oestrous cycle. The expression of both steroid receptors have been reported to vary during the oestrous cycle, based on immunohistochemical studies in sheep (Zhao *et al*., 1999), dogs (Vermeirsch *et al*., 1999; Vermeirsch *et al*., 2000) and rats (Wang *et al*., 2000) or biochemical studies in cows (Vesanen *et al*., 1991; Vesanen *et al*., 1993) and horses (Re *et al*., 1995). On the other hand, no clear relationship could be found between the stage of the cycle and immunohistochemical steroid receptor expression in women (Kuprija□czyck and Möller, 1988; Cano *et al*., 1990; Kuprija□czyck, 1991; Snijders *et al*., 1992).

To date, there have been reports on many possible factors, that might be involved in cervical softening during the oestrous phase. Surprisingly, relatively few studies explored the possibility that structural changes in the stromal connective tissue might be responsible for the change in texture between the oestrous phase and the rest of the cycle (Petersen *et al*., 1991; Rimmer *et al*., 1972) or dealt with the effect of oestrogen on collagen biochemistry (non-pregnant pigs: Hall and Anthony, 1993; Huang *et al*., 1997). Because these reports were also not uniform in their conclusions, we decided to investigate this possibility for the cow, by analysing the changes in collagen biochemistry of the cervix during the oestrous cycle, before we went on to look at changes in the cervix of pregnant and parturient cows. Therefore, we collected cervical tissues from two groups of non-pregnant cows, one with high (P4 > 2.0 ng/ml) and the other with low progesterone (P4 $\leq$ 0.5 ng/ml) concentrations in the peripheral circulation. Collagen content, percentage of collagen denaturation and water content were measured in **chapter two** and we also performed immunohistochemistry to study the expression of both oestrogen receptor$\alpha$ (ER$\alpha$) and progesterone receptors (PR) in two similar groups of cows (**chapter three**). The cows that were under low progesterone influence had significantly higher tissue concentrations of oestrogens in the cervix than the cows under high progesterone influence (chapter 3), which justified the conclusion that these animals were indeed under high oestrogen influence. We also found that the differences in texture between the cervices of the two groups with different progesterone status could not be explained by differences in collagen characteristics. However, we did observe several differences between the superficial and deep stromal layer of the cervix.

Significant differences in collagen degradation and water content were present between the superficial and the deep stromal layer, while both layers had similar collagen contents. This indicates that differences in collagen degradation and water content may explain the differences in texture between the two layers (chapter 2). In addition, the significant differences in cell density between the two layers that was found (chapter 3), may also be a cause of the difference in texture. We also observed an increasing gradient of receptor expression from epithelium through superficial to deep stromal layer, which was further enhanced by the lower cell density of the superficial stroma when compared to the deep stromal layer. These findings, along with the observation that the superficial stromal layer contains almost no smooth muscle cells, in contrast to the deep stromal layer (Breeveld-Dwarkasing *et al*., 2000), indicate that these two layers have different functions and are differently regulated.

The observation that there were no differences in ER and PR expression between the two groups of animals with different progesterone status, indicated that hormonal action might not only be regulated through the expression levels of their receptors. In fact, due attention should be given to the presence of natural antagonists, receptor-activators and repressors, or other receptor isoforms that were not included in our histochemical analysis (Klinge, 2000; Peach *et al*., 1997; Wang *et al*., 2000).

We also looked if there were differences in biochemical collagen characteristics, along the longitudinal axis of the cervix, because differences in the degree of softening and speed of dilatation along the longitudinal axis have been reported at parturition in sheep (Ledger *et al*., 1985) and pigs (O'Day *et al*., 1989; O'Day-Bowman *et al*., 1991; Winn *et al*., 1993). We found that the uterine end of the non-pregnant cervix had a significantly lower collagen content than the vaginal end (chapter 2). However, at this time we do not know if the same is true for the cervix during pregnancy and we have no explanation on how this might affect the speed of dilatation. It seems obvious that the uterine end of the cervix has to soften earlier than the vaginal end during parturition, although the uterus itself has no influence on softening, as Ledger *et al*., (1985) showed by surgically separating the cervix from the uterus in pregnant sheep. We also found that the immunohistochemical expression of both ER and PR at the uterine end was significantly lower than at the vaginal end. However, these differences were no longer present when a correction was made for the fact that the uterine end of the cervix had a higher cell density than the vaginal end (chapter 3). At this time, it is not clear how this difference would influence the local effect of the two steroid hormones on the cervix.

Although we could not find any differences in collagen content, percentage of collagen denaturation and water content or differences in the expression of the ER and PR, between the cows with high progesterone and the cows with low progesterone status, we did find that significant regional differences are present in the non-pregnant cervix. Account should be given to these regional differences when studying structural changes in the connective tissue or changes in steroid hormone receptor expression, before conclusions can be made with regard to the effect of endocrinological status (luteal, oestrous or pregnant) on the functional state of the cervix.

## Cervical softening during pregnancy and parturition

During pregnancy, the cervix has to remain firm and closed until the foetus is fully developed and ready to be born. On the other hand, the cervix also has to prepare itself to be stretched open so that the foetus can pass during delivery. This means that considerable structural changes have to take place in the connective tissue of the cervix, that need to be conspicuously timed. In contrast to former thoughts, that final cervical softening (remodeling) was the result of pressure from uterine contractions on the cervix, there is enough convincing information available that softening of the cervix does not depend on the uterus (Hollingsworth and Gallimore, 1982; Ledger *et al*., 1985; Owiny *et al*., 1992). A change in cervical structure already starts at least at the beginning of the third trimester of gestation, as has been shown in several species such as the rat (Harkness and Harkness, 1959; Rimmer, 1973; Shi *et al*., 1999), sheep (Fitzpatrick, 1977), cow (Kaidi *et al*., 1995). However, the findings of Owiny *et al*. (1991) in sheep, that these changes only result in a small increase in compliance of the cervix, while as late as a few

hours before parturition the compliance is dramatically increased, indicate that shortly before cervical dilatation, additional structural changes yet have to take place. Most authors are aware of the fact that different cellular mechanisms must be responsible for cervical ripening on the one hand and the more dramatic softening during parturition. For example, in contrast to their almost complete absence during early and term pregnancy, a significantly increased presence of leucocytes during parturition has been reported for several species (Owiny *et al*., 1995; Yoshida and Manabe, 1990; Knudsen *et al*., 1997; Winkler *et al*., 1999; Luque *et al*., 1996). Similarly, a significant increase in collagenolytic enzymes is found only during parturition (Rajabi *et al*., 1991; Granström *et al*., 1992). These findings indicate that catabolic processes predominate during labour, while more anabolic processes may take place during ripening (Winkler and Rath, 1999; Leppert, 1995). However, studies that have focussed on collagen changes in the cervix, report that a decrease in collagen content and/ or concentration took place between early pregnancy and term pregnancy (Danfort and Buckingham, 1964; Uldjberg *et al*., 1983; Stjernholm *et al*., 1996; Granstrom *et al*., 1989: for women; Fitzpatrick, 1977; Regassa and Noakes, 2001: for sheep; Kaidi *et al*., 1995: for cows), rather than an increase which one might expect from anabolic processes. An increase of the water content or of other cervical proteins has been proposed to be responsible for this apparent contradiction. It has also been suggested that newly synthesised collagen is already degraded by cervical enzymes, before it is cross-linked into the collagen network (Leppert, 1995).

An important factor, that has so far inhibited to differentiate between cervical ripening and final softening of the cervix, on the basis of biochemical analysis of the connective tissue changes, is the fact that it was hardly possible to obtain biopsy samples during subsequent periods within one pregnancy. In **chapter four**, we report on the successful use of repeated collection of biopsy samples from pregnant cows, to study changes in the collagen biochemistry at three different moments during gestation.

Another factor, which may also be responsible for some of the apparent contradiction, is the fact that until now collagen degradation has not been differentiated from any further digestion of collagen molecules, by for example non-specific proteolytic enzymes. Therefore, we used a test that was validated in human cartilage (Bank *et al*., 1997), to differentiate the percentage of collagen denaturation, as a quantitative parameter of collagen degradation, from possible collagen loss as expressed by decreasing collagen content (μg/mg dry weight) and/ or concentration (μg/mg wet weight) (chapter 2 and 4).

Our findings for the pregnant cow lead us to assume that cervical ripening is a gradual process that takes place between mid gestation and term pregnancy and is typified by a significant increase in synthesis of collagen, combined with a significant increase in collagen degradation. Between term pregnancy and calving, collagen loss predominates because a significant decrease in both collagen content and concentration was found, but no further increase in collagen degradation occurred between the two stages. The above mentioned cellular mechanisms (leucocyte infiltration, collagenolysis) that indicate an increase in

catalytic processes during labour when compared to earlier gestation, may explain the collagen loss as observed in chapter 4.

Based on our findings reported in chapter 2, we separately analysed the superficial and the deep stromal layer of all the biopsies that were obtained for the study in chapter 4. In accordance with the data of the non-pregnant cervix (chapter 2), the superficial and deep stromal layer of the pregnant and parturient cervix did not differ in collagen content and concentration, but we did observe that characteristics of cervical ripening are more pronounced in the deep stromal layer than in the superfical stromal layer. The findings in chapter 2 and 4 emphasise the need for a detailed description of the origin of the tissue samples when studying biochemical and cellular aspects of cervical function.

## Cervical dilatation related to uterine activity and endocrinological changes

Shortly before and even during parturition, dramatic changes in the structure of the cervical connective tissue take place, probably in a time span of 24-48 hours in the case of cows, and maybe even during a shorter interval in species with shorter gestational lengths. The many cellular factors that are involved in this process are inflammatory-associated and these potentially destructive elements need to be carefully regulated. More insight in the timing of these factors, for example, would be beneficial in the management of protracted labour or induction protocols in women (Granström *et al*., 1991; Stempel *et al*., 1997) or other species such as horses (Rigby *et al*., 1998). It would greatly enhance our understanding of cervical dilatation, if we were able to judge these functional processes relative to the start/ stage of parturition rather than to gestational age. Following from this, pharmacological protocols might be more tailored to the stage of parturition, or decisions for surgical interference could be made earlier in case of unsuccessful induction protocols. Because the period in which the cascade of final changes takes place is relatively short compared to the rest of gestation, it is hardly possible to obtain biopsies that can be accurately timed, if the mothers are already in spontaneous parturition. Therefore, for this type of investigation an experimental model is required, that mimics spontaneous parturition, and enables us to identify specific landmarks of parturition and in the meantime allows us to obtain repeated biopsies during these periods of interest.

It has been possible to continuously measure cervical dilatation in women during labour by using ultrasound cervimetry, combined with intrauterine pressure (IUP) recordings (Eijskoot *et al*., 1976; Kok *et al*., 1977, van Dessel *et al*., 1994). In **chapter five** it was described how this technique was adapted for the cow. We were therefore able to measure the dilatation of the caudal cervix continuously until the beginning of expulsion in cows, after induction of parturition with a synthetic analogue of $PGF_{2\alpha}$. However, in this study the quality of simultaneously made IUP recordings (by means of transcervically introduced pressure catheters), proved to be insufficient to continuously monitor and quantify uterine activity. Therefore, it

was not possible to relate onset and progress of cervical dilatation to characteristics of uterine activity.

The dilatation curves that we obtained in the study of chapter 5 were analysed to find the moment that dilatation of the caudal cervix started. We used the classical Friedman curve (Friedman 1955; Friedman 1956) for cervical dilatation in women, as a reference model. According to Friedman, cervical dilatation is divided in four stages: 1) the latent phase, during which no appreciable dilatation takes place, 2) the acceleration phase, marked by a rapid change in the slope of the dilatation curve 3) the phase of maximal slope, with a linear and rapid dilatation and 4) the deceleration phase during which dilatation slows down considerably compared to the phase of maximal slope. We demonstrated that these four phases could not always be recognised in the parturient cow.

In the subsequent study (**chapter six**), uterine EMG recordings, instead of IUP measurements, were used to quantify uterine activity during cervimetry recordings. Because in the former study (chapter 5), it became clear that cervical dilatation in cows did not always conform to the Friedman curve, a two-compartment non linear regression model was used to find the transition points between the latent phase and the dilatation phase, assuming linear progression in both phases. The same model was used to identify the transition from the state of uterine quiescence to the beginning of uterine activity. We started the cervimetry/ EMG recordings at the moment that the animals were given the luteolytic PG injection and we measured changes in the maternal plasma concentrations of oestrogen, progesterone and 15-ketodihydro-$PGF_{2\alpha}$ -metabolite (PGFM). This way, we were able to identify a well predictable sequence of physiological changes in maternal hormone concentrations, myometrial activity and dilatation of the caudal cervix. This sequence can be summarised as follows:

1. Within 4 hours after the PG injection a significant drop in the maternal plasma progesterone concentrations took place, and gradually decreased further until around 1.0 ng/ml, remaining there until calving.
2. Meanwhile, plasma oestradiol-17$\beta$ concentrations increased, until significantly elevated from 28 h after the treatment and further.
3. Eight hours after PG, plasma PGFM concentrations began to increase also, and reached significantly elevated levels at 20 h after PG.
4. Immediately after PG injection, there was an approximately 4 h period of increased myometrial activity, which was followed by several hours of almost complete myometrial quiescence. Thirteen hours after the PG injection myometrial activity began to increase again.
5. Fifteen and a half hours after the onset of myometrial activity, cervical dilatation started with an overall mean dilatation rate of 2.25 cm/ h. Some 9 h later, expulsion of the calf started.

From the results in chapter 6 and by others (Janszen *et al*., 1990; Janszen *et al*., 1993; Koets *et al*., 1998) it is concluded that induction of parturition with $PGF_{2\alpha}$ in late pregnant cows forms a well-manageable, protocol which closely

resembles spontaneous parturition (Kundig *et* al., 1990) and can be of great value for future studies on functional changes in the cervix during parturition.

## Future prospects

In this thesis we have focussed on biochemical characteristics of collagen within the stromal layer, 1) to explain for differences in texture of the cervix between two groups of non-pregnant cows with different progesterone status and between different regions of the cross-sectional and longitudinal axis of this organ and 2) to explain differences between cervical ripening and the final softening that occurs during parturition. We realise, however, that collagen is not the only connective tissue component in the cervix and that the texture of this tissue may also be influenced by the content and composition of the glycosaminoglycan component of the cervix. In fact, fluctuations in the concentration of oestrogen and progesterone, such as occur during the oestrous cycle and at parturition may influence the composition and the concentration of the glycosaminoglycan component of the connective tissue (Tanaka *et al*., 1997). Further studies are neccesary, to assess if glycosaminoglycans could play a role in explaining differences in texture between the oestrous phase and the rest of the cycle. In addition, differences in glycosaminoglycan content and composition may also help to explain the differences we found in collagen content along the longitudinal axis.

We assume that the increased collagen content at term pregnancy is the result of a gradual process that took place between mid gestation and term pregnancy instead of a suddenly increased synthesis of collagen shortly before term. The latter would lead to a highly increased amount of newly synthesised and non-cross linked collagen and these fragments would have been removed from the samples during treatment with guanidium hydrochloride (GuHCl), before we measured collagen content. Such an increased amount of non cross-linked collagen would have resulted in a decreased collagen content, rather than in the significant increase that we found. However, we have not undisputedly proven this to be the case. Low pentosidine levels can be an indication of a high rate of collagen synthesis, because they are also a measure of less cross-links (Bank *et al*., 1999). It could be interesting to include this measurement in further studies on collagen changes of the parturient cervix to differentiate between degenerated old collagen and newly synthesised collagen.In addition to collagen there is an important role for glycoseaminoglycans (including proteoglycans) during cervical ripening and final softening. The most dramatic changes in content and composition of the glycosaminoglycan component have been reported to occur between term pregnancy and labour (Fossang *et al*., 1984; Osmers *et al*., 1993). Hyaluronic acid does not only contribute to the mechanical properties of the cervix, it may also play a role in the final cervical ripening by stimulating $PGE_2$ synthesis and cytokine production in humans (Kobayashi *et al*., 1998). Similarly, heparan sulphate has been shown to enhance the leucocyte attraction by interleukin-8 in the cervix of rabbits (Belayet *et al*., 1999).

Oestrogens either directly or indirectly enhance the cytokine stimulated attraction of leucocytes to the cervix while this effect may be blocked by progesterone (Tanaka *et al*., 1997; Ramos *et al*., 2000). Recently ERβ was shown to be present in leucocytes in the human cervix, which suggests that oestrogens may also directly regulate leucocyte functions (Stygar *et al*., 2001), such as the release of metalloproteinases and toxic metabolites. It would be interesting to investigate the relationship between the occurrence of these cytokine mediated effects in the connective tissue of the cervix and the changes in oestrogen/ progesterone ratios in the maternal blood during parturition, such as we found for the cow in the study described in chapter 6.

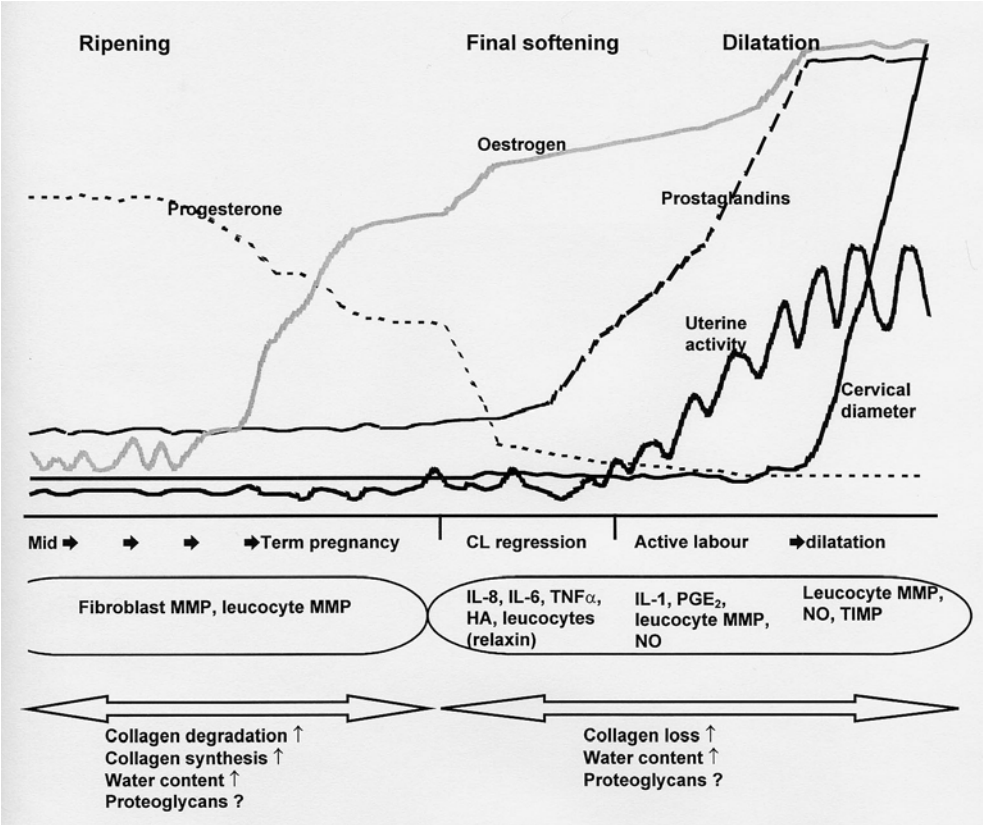


Figure 1. Proposed model of functional changes in the bovine cervix uteri during pregnancy and parturition, in relation to uterine activity and hormonal changes in the maternal circulation. Included are some of the cellular and biochemical mechanisms that might be of interest for future studies.

The experimental model as presented in chapter 6 opens up possibilities for more detailed studies on several cellular mechanisms underlying the final cervical softening, when it is combined with the biopsy technique described in chapter 4. In fact, if we extrapolate the description of the temporal changes of maternal plasma interleukin-1 concentrations in cows by Koets *et al*. (1998), who used the same induction protocol as we did, it is intriguing that this cytokine starts to increase during the period of relative myometrial quiescence and almost linearly progresses with the increase of uterine activity, to reach maximum levels around 22 h after PG, which is more than 6 hours before we recorded the onset of cervical dilatation (chapter 6). These findings, also support the hypothesis by Janszen *et al*. (1993), that the period of myometrial quiescence allows the cervix to prepare for final dilatation. In Figure 1, a model of functional changes in the cervix during pregnancy and parturition based on our findings in chapters 2–6 is proposed, including some of the cellular mechanisms that might be of interest for further studies.

Of course, we are also aware of the fact that we only recorded the dilatation at the vaginal end of the cervix and that dilatation at the uterine end may already have started before we were able to record the first excursions of the cervical diameter at the vaginal end. However, frequently repeated trans-rectal ultrasound scanning of the cervix in PG induced calvings (personal observation) have shown that the cranial part of the cervix starts to dilate within less than 4 hours before the caudal part starts to dilate. Further study may shed some more light, on how changes at these two ends of the cervix are temporally related with each other.

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# Samenvatting

In dit proefschrift komen verschillende aspecten ter sprake die te maken hebben met functionele veranderingen in de cervix uteri van het rund. Er zijn twee verschillende stadia binnen de reproductie cyclus van het vrouwelijke dier of de mens, waarin de morfologische kenmerken van de cervix veranderen. Tijdens de oestrische fase van de cyclus, is de cervix bij vele species zachter dan tijdens de rest van de cyclus en kan zelfs enigszins geopend zijn. Gedurende de rest van de cyclus is de cervix in het algemeen veel meer rigide en nauwelijks te penetreren. Tijdens de zwangerschap ondergaat de cervix drastische veranderingen. Gedurende het grootste deel van de zwangerschap is de cervix stijf en goed gesloten, om te voorkomen dat de zich ontwikkelende foetus in contact komt met de schadelijke buitenwereld. Echter, op het moment dat de partus plaats vindt, wordt de cervix een rekbare structuur, waarvan de diameter van het lumen vele malen wordt vergroot om zodoende de volgroeide foetus geboren te kunnen laten worden. Reeds tijdens de zwangerschap wordt de cervix zachter, hetgeen rijping of maturatie genoemd wordt, ter voorbereiding op de meer dramatische verslapping van het weefsel, tijdens de partus.

De veranderingen die plaatsvinden in de cervix tijdens de oestrische fase van de cyclus, tijdens de zwangerschap en uiteindelijk de partus, vinden voornamelijk plaats in het stroma van de cervix. Het stroma van de cervix is bovendien te verdelen in een oppervlakkige laag die zich onderscheidt van het diepe stroma door een lagere cel dichtheid, een enigszins verschillende weefsel samenstelling en een zachtere consistentie. Het stroma is voor een zeer groot deel opgebouwd uit bindweefsel en collageen is daarvan een van de grootste bestanddelen. Collageen bindweefsel bestaat uit een driedimensionaal netwerk van eiwit moleculen met een triple helix structuur, die door onderlinge crosslinks een stevig en rigide geheel vormt. De triple helix structuur maakt de moleculen zeer bestand tegen afbraak (digestie), behalve als de triple helix ontwonden (gedenatureerd ofwel gedegradeerd) raakt. In dat geval zijn de ontwonden uiteinden van het molecuul gemakkelijk te digesteren door verschillende a-specifieke proteinases. Een bepaalde categorie proteolytische enzymen, die onderdeel uitmaken van de matrix metalloproteinases zijn verantwoordelijk voor het denaturatie proces. De gedenatureerde moleculen, maar nog niet gedigesteerde moleculen blijven door de coss-links nog wel geïncorporeerd in het drie dimensionale netwerk, waarvan de mechanische eigenschappen overigens wel zijn veranderd. Behalve collageen bevindt zich in de interstitiële ruimte verschillende glycosaminoglycanen, die bijdragen aan de visco-elastische eigenschappen van het bindweefsel. Daarnaast bestaat het stroma voor ongeveer 80% uit water. Veranderingen in deze drie compartimenten leiden tot structuur veranderingen in het stromale bindweefsel, met als gevolg veranderde visco-elastische kenmerken van de cervix.

Het eerste deel van het proefschrift beschrijft een onderzoek van de cervix van niet drachtige runderen. Van twee groepen runderen die onder verschillende progesteron invloed stonden, werden de cervices op het slachthuis verzameld. De

groep met een hoge progesteron status hadden een perifere plasma progesteron concentratie van > 2.0 ng/ ml, de groep met een lage progesteron status hadden een plasma concentratie van $\leq$ 0.5 ng/ ml. De laatste groep bleek ook een significant hogere oestrogeen concentratie in het cervix weefsel te hebben dan de groep met een hoge progesteron status, hetgeen de conclusie rechtvaardigt dat deze dieren onder hoge oestrogene invloed stonden. In het onderzoek dat in **hoofdstuk twee** van dit proefschrift wordt beschreven werd allereerst de vraag gesteld of veranderingen in de biochemische eigenschappen van het collageen mogelijk verantwoordelijk waren voor de verschillen in consistentie van de cervix van het rund tijdens de oestrus fase vergeleken met de cervix in de luteale fase. Hierbij werden collageen degradatie (gemeten als percentage denaturatie) en collageen gehalte: (in $\mu$g/ mg droog gewicht) als parameters gebruikt. Tevens werd onderzocht of de verschillen in consistentie tussen de oppervlakkige en de diepe stromale laag verklaard konden worden door verschillen in deze biochemische kenmerken van het collageen. Omdat er in de literatuur ook wordt beschreven dat tijdens de partus de cervix zich niet langs de gehele lengte in dezelfde mate en met dezelfde snelheid blijkt te verslappen werd er ook onderzocht of er verschillen in de biochemische kenmerken van het collageen langs de longitudinale as van de cervix bestonden, die hier mogelijk voor verantwoordelijk konden zijn.

Omdat progesteron en oestrogeen een regulerende invloed hebben op het evenwicht tussen anabolische en katabolische activiteit in het stromale bindweefsel van de cervix, werd in **hoofdstuk drie** onderzocht met immunohistochemie in hoeverre de expressie (aantal positief gekleurde kernen per 1000 cellen) van de progesteron receptor en oestrogeen receptor-$\alpha$ verschilde, tussen runderen met een hoge progesteron en runderen met een lage progesteron status. Ook hier werd onderzocht of er verschillen voorkomen in de expressie van de twee receptoren tussen verschillende weefsellagen langs de circulaire as en langs de longitudinale as van de cervix.

Noch in de biochemische kenmerken van het collageen, noch in de immuunhistochemische expressie van de twee steroïd hormonen, werden er verschillen gevonden die gerelateerd waren aan de progesteron status van het dier. Echter, er werd langs de gehele longitudinale as in de oppervlakkige stroma laag, een significant hogere mate van collageen degradatie gevonden dan in de diepe stroma laag, terwijl beide een gelijk collageen gehalte hadden. Tevens werd in de oppervlakkige laag een significant hoger water gehalte gevonden dan in de diepe laag. Aan het vaginale uiteinde van de cervix werd bovendien een significant hogere collageen gehalte gemeten dan aan het uterine uiteinde.

De expressie van de oestrogeen receptor-$\alpha$ en die van de progesteron receptoren waren sterk positief gecorreleerd met elkaar en voor beide receptoren goldt dat de expressie in radiale richting significant toenam (dus van het epitheel via de oppervlakkige stroma laag naar de diepe stroma laag). Het verschil tussen de oppervlakkige laag en diepe laag werd nog eens versterkt door een significant verschil in cel dichtheid tussen de twee lagen. De expressie van beide steroid receptoren was ook significant hoger aan de uteriene zijde dan aan de vaginale zijde, maar in dit geval werd het verschil tenietgedaan wanneer gecorrigeerd werd

voor het feit dat het stroma aan de uteriene zijde een significant hogere cel dichtheid had dan de vaginale zijde.

Het eerste deel van dit proefschrift leidt tot de conclusie dat 1) het zachter worden van de cervix tijdens de oestrische fase van de cyclus niet gepaard gaat met veranderingen in de mate van degradatie van het collageen of met veranderingen in het collageen en water gehalte van de cervicale stroma en 2) dat er ook geen relatie lijkt te bestaan met de immuunhistochemische expressie van progesteron en oestrogeen receptoren. Echter, de verschillen die gevonden werden langs de radiale en de longitudinale as van de cervix nopen tot een zorgvuldige beschrijving van het gebied van de cervix dat bemonsterd wordt bij studies naar cellulaire en biochemische veranderingen in de cervix.

Het tweede deel van het proefschrift beschrijft de functionele veranderingen in de cervix van het drachtige en in partus zijnde rund. Het onderzoek aan de cervix wordt in het algemeen belemmerd door ethische en praktische bezwaren. In de meeste species is het bijvoorbeeld niet mogelijk om longitudinale studies te verrichten naar weefselveranderingen omdat dit zou inhouden dat er meerdere malen biopten verzameld zouden moeten worden van de cervix. Voor zwangere vrouwen wordt dit als zeer belastend ervaren en bij de meeste diersoorten (rat, cavia, konijn, schaap) die tot nu toe veelvuldig als model werden gebruikt is het vaak praktisch onmogelijk om een cervixbiopt te verkrijgen, laat staan dat dit herhaald zou kunnen worden bij hetzelfde dier. Weefsel monsters worden daarom meestal verkregen tijdens gynaecologische operaties (vrouwen) of door het dier op te offeren. De locaties binnen de cervix waar men de monsters vandaan haalt verschillen vaak tussen diverse studies. Het gevolg is dat er eigenlijk altijd cross-sectional studies worden uitgevoerd, waarbij gemiddelde waarden van groepen met elkaar vergeleken worden. Een probleem dat zich hierbij voordoet is, dat om deze groepen te formeren, gekeken wordt naar de lengte van de draagtijd in plaats van het stadium ten opzichte van de partus. Echter, vlak vóór of tijdens de partus vinden de meest in het oog springende veranderingen plaats in de cervix, die waarschijnlijk ook zeer scherp in de tijd gebonden zijn ten opzichte van de uterus contractiliteit en die gereguleerd worden door de hormonale veranderingen vlak voor en tijdens de partus. Dit impliceert dat als men cellulaire en biochemische veranderingen in de cervix wil bestuderen, het stadium van de zwangerschap ten opzichte van de uiteindelijke partus van doorslaggevend belang is. Door individuele variaties in de draagtijd, die bij sommige species relatief groot kunnen zijn, kan de spreiding in de groepen bij een cross-sectional opzet van een studie enorm groot zijn.

In **hoofdstuk vier** werd een studie verricht naar de veranderingen in collageen eigenschappen (collageen degradatie: percentage denaturatie; collageen gehalte: μg/ mg droog gewicht; collageen concentratie: μg/ mg nat gewicht) bij het rund, tijdens de dracht en de partus. In deze studie werd een longitudinale opzet gevolgd door bij hetzelfde dier op drie momenten biopten aan de vaginale zijde van de cervix te verzamelen. Biopten werden verzameld halverwege het tweede trimester van de dracht (gemiddelde draagtijd van het rund is 280 dagen), een tweede biopt werd verzameld op exact 275 dagen dracht (á

terme) en het derde biopt werd zo snel mogelijk na het spontaan afkalven verzameld. Naar aanleiding van de resultaten in de hoofdstukken 2 en 3 werden de biopten bovendien steeds verdeeld in een oppervlakkige stroma deel en een diep stroma deel. De conclusie uit deze studie was dat een geleidelijke rijping van de cervix die plaats vindt tussen het tweede trimester en á terme gekenmerkt wordt door een toename in de collageen synthese (afgeleid uit een significant toegenomen collageen gehalte en concentratie van de á terme cervix vergeleken met het tweede trimester) en tegelijkertijd een significante toename in de mate van degradatie van het collageen (toegenomen percentage denaturatie). De verslapping van de cervix die tijdens de partus plaatsvindt wordt echter gekenmerkt door een verlies van collageen (afgeleid uit een significante daling in het collageen gehalte en de collageen concentratie van de cervix na het afkalven vergeleken met de á terme cervix). Dit wordt waarschijnlijk veroorzaakt door een toename in de verdere digestie van de reeds gedenatureerde collageen moleculen tot kleine oplosbare fragmenten. Het is mogelijk dat andere proteolytische enzymen dan die welke verantwoordelijk waren voor de toegenomen degradatie, hiervoor verantwoordelijk zijn. Ook bleek dat de veranderingen die gepaard gingen met de rijping en de daaropvolgende verslapping van de cervix voornamelijk in de diepe stromale laag plaats vinden. De laatste bevinding benadrukte nogmaals de noodzaak tot een gedetailleerde beschrijving van de aard van het biopt of weefsel monster dat onderzocht wordt, om de resultaten op de juiste wijze te kunnen interpreteren.

Met het in hoofdstuk 4 beschreven biopterings protocol werd geïllustreerd dat door á terme een gefixeerde dag van de dracht als referentie te kiezen ten opzichte van de monsters die verzameld werden na het afkalven, er een spreiding van 2-17 dagen zat in het interval tussen het tweede en derde biopt. Het is echter praktisch onmogelijk om het tijdstip van biopteren zodanig te protocolleren dat ze steeds in hetzelfde stadium zitten ten opzichte van het afkalven, als gebruik wordt gemaakt van spontaan afkalvende dieren. Bij runderen is het mogelijk de partus te induceren door middel van het opwekken van luteolyse met behulp van een synthetisch $PGF_{2\alpha}$ bij het á terme drachtige dier. Dit leidt tot hormonale veranderingen en een partus verloop die zeer goed vergelijkbaar zijn met het verloop tijdens een spontane partus. Wanneer de uterus contracties en de hormonale veranderingen gerelateerd zouden kunnen worden aan het verloop van de ontsluiting van cervix, zou het mogelijk moeten zijn om cellulaire en biochemische veranderingen die gemeten worden in cervix biopten, verkregen tijdens de laatste fase van de zwangerschap, gerelateerd kunnen worden aan het stadium van de partus, afgemeten aan de hormonale veranderingen en weeën activiteit en aan de mate van ontsluiting. In **hoofdstuk vijf** werd daarom onderzocht of met behulp van ultrasone cervimetrie het verloop van de cervix dilatatie bij koeien, waarbij de partus geïnduceerd werd met behulp van $PGF_{2\alpha}$, continu geregistreerd kon worden. Hiertoe werden twee ultrasound transducers (één als zender en de ander als ontvanger van het ultrageluidssignaal) tegenover elkaar bevestigd aan de opstaande rand van de portio vaginalis van de cervix, waardoor de veranderende diameter van de caudale cervix opening, continue kon worden geregistreerd. Deze techniek die al eerder met succes bij barende vrouwen

was toegepast werd aangepast voor gebruik bij het rund. Het bleek zeer goed mogelijk om gedurende lange tijd, tot aan het begin van de uitdrijvingsfase, de dilatatie van de cervix te registreren. Er moest echter ook geconcludeerd worden dat het registreren van de intra-uteriene drukveranderingen door middel van een tegelijkertijd, via transcervicale weg ingebrachte drukkatheter bij het rund, onvoldoende goed functioneerde om de uterus activiteit te kunnen relateren aan de ontsluiting van de cervix. Daarom werd in **hoofdstuk zes** de cervimeter gecombineerd met de registratie van de elektrische activiteit van het myometrium. Hiertoe werden tijdens een laparotomie EMG elektroden aan het uterus oppervlak bevestigd. Door middel van catheterisatie van de arteria circumflexa, was het tevens mogelijk om gedurende langere tijd meermalen bloedmonsters te verzamelen en zodoende veranderingen in de plasma concentraties van progesteron, oestradiol-17$\beta$ en 15-keto-dihydro-prostaglandine-metabolieten, tijdens de partus te kunnen vervolgen. Hierdoor was het mogelijk om de temporale veranderingen in hormoon concentraties in het maternale bloed en de uterus activiteit in relatie tot het ontsluiten van de cervix voor het rund, in detail te beschrijven. De volgorde waarin de veranderingen plaats vonden kan alsvolgt worden samengevat:

1. Binnen 4 u na de prostaglandine injectie vond er een significante daling van het plasma progesteron gehalte in het maternale bloed plaats. De concentratie daalde daarna geleidelijk tot een niveau van ongeveer 1 ng/ml en bleef daar tot aan het afkalven.
2. Tegelijkertijd steeg de concentratie van oestradiol-17$\beta$ in het maternale bloed en bereikte vanaf 28 u na de injectie een significant hogere concentratie dan ervoor.
3. Acht uur na de prostaglandine injectie, begon de concentratie van het 15-keto-dihydro-$PGF_{2\alpha}$ metaboliet in het maternale plasma geleidelijk te stijgen tot significant verhoogde waarden, vanaf 20 u na de injectie.
4. Meteen na de prostaglandine injectie was er een periode van ongeveer 4 u, waarin er sprake was van verhoogde myometrium activiteit die vervolgens bijna volledig verdween. Dertien uur na de injectie begonnen de uterus contracties weer geleidelijk toe te nemen.
5. Vijftien en een half uur na het op gang komen van de uterus contracties begon de ontsluiting aan de caudale zijde van de cervix met een gemiddelde ontsluitingssnelheid van 2.25 cm/ u. Gemiddeld 9 u na de start van de ontsluiting begon de uitdrijvingsfase .

Het model beschreven in hoofdstuk 6, biedt in combinatie met de in hoofdstuk 4 beschreven biopsie techniek de mogelijkheid om gedetailleerde longitudinale studies te verrichten naar cellulaire en biochemische veranderingen in de cervix, gerelateerd aan het stadium van de dracht of partus, in plaats van met “cross-sectional” studies zoals die tot nu toe bij andere diersoorten werd uitgevoerd.

# Dankwoord

En dan is het boekje klaar. Velen ben ik dank verschuldigd, want in mijn eentje had ik dit niet gekund. Bovenal, dank ik mijn Hemelse Vader voor de bijzondere kans die Hij mij gaf en voor de vele mensen die hij op mijn pad bracht. Het zijn er veel zoals ik al zei, en ik kan niet iedereen bij naam noemen. Toch zijn er een aantal mensen wie ik bijzonder dankbaar ben voor hun bijdrage aan de totstandkoming van dit proefschrift die ik persoonlijk wil noemen.

Marcel Taverne, je begon als mijn co-promotor en eindigt als mijn promotor. Je stempel op dit proefschrift en mijn beginnende wetenschappelijke carriere is onmiskenbaar. Als laatstejaars student kwam ik bij je langs en sindsdien heb je mij op alle mogelijke manieren gestimuleerd om verder te gaan in het onderzoek. Ik dank je voor je vertrouwen in mijn kunnen en mede dankzij jou kritische maar altijd stimulerende commentaar op al mijn schrijfsels is dit boekje wat het geworden is.

Bert van der Weijden, je grote enthousiasme voor de verloskunde heb ik altijd bewonderd, want je was niet alleen mijn promotor maar ook mijn begeleider op het pad der edele verloskunde. Ik heb veel van je geleerd op beide vlakken en ben daar erg dankbaar voor.

Federica van Dissel-Emiliani, ik vond het erg fijn om met jou te mogen werken. Behalve als co-promotor beschouw ik jou ook als mijn vriendin op het hobbelige promotie pad. Je hebt mijn ideeën altijd serieus genomen en mij veel vrijheid gegeven in de inhoud van de artikelen. Waar nodig heb je mij bijgestuurd. En daarnaast heb ik ook genoten van onze andere gesprekken, het zo af en toe mijmeren over Italië, Suriname of andere warme oorden.

Fred Lotgering, mijn tweede co-promotor. Het was soms wel een grote overbrugging die gemaakt moest worden van de vrouw naar de koe en omgekeerd - en dit leidde soms wel tot wat spraakverwarring. Toch vergeet ik niet de dag dat je voor het eerst keer de cervix van een koe palpeerde, of moet ik zeggen exploreerde, en je zei:" Het verschilt eigenlijk helemaal niet zoveel van een vrouw!". En daarmee sloeg je de spijker op zijn kop, want dat is toch minstens één van de essenties van dit proefschrift. Dank je voor het enthousiasme dat je gestopt heb in de voorbereiding van de cervimetrie experimenten, je stevige kritiek, maar ook voor het feit dat je open stond voor mijn ideeën.

Piet Struijk, voor mij ben je onmisbaar geweest. Wat zou ik doen zonder jouw technische vernuft en statistische kennis. Je was altijd bereid te helpen, zelfs op je vrije zondag kwam je gewoon, als er iets mis was met de meet apparatuur. Het was gewoon een plezier en ontzettend gezellig om met jou te mogen werken.

Frits Eijskoot, dankzij jouw cervimeter konden wij twee prachtige experimenten toevoegen aan dit boek.

Mieke de Boer, vooral in het begin was je mijn steun en toeverlaat bij het ingeburgerd raken op het celbiologie lab. De fijne kneepjes van de histochemie en immuunhistochemie heb ik van jou geleerd. Ook een ieder die werkt op de hoofdafdeling Biochemie, Celbiologie en Histologie wil ik bedanken voor de vele hulpjes en voor de prettige sfeer waardoor ik mij thuis voelde op de afdeling, ondanks dat ik er niet de hele tijd was.

Johan Tekoppele en Ruud Bank van TNO-PG te Leiden, heel erg bedankt voor jullie gastvrijheid op het lab en de fijne weetjes op het gebied van collageen biochemie. En natuurlijk ook de anderen die op het lab werkten en werken, bedankt voor de hulp.

Riek van Oord, Csaba Baicsy en Rineke de Jong, heel veel dank ben ik jullie verschuldigd. Bedankt voor jullie hulp met de voorbereiding van de cervimetrie experimenten. Dank zij jullie kon ik toch nog een beetje slapen tijdens de soms dagenlange cervimetrie experimenten. En met een gerust hart; omdat ik wist dat ik het aan jullie kon overlaten.

Nicoline Soede hielp met de statistiek van drie artikelen, waarvoor mijn grote dank.

Het OK personeel, in het bijzonder Frans van Kooij en Henk Heuveling, bedankt voor jullie assistentie en gezelligheid tijdens de operaties van proefdieren en patiënten. Alle dierverzorgers ben ik ook dankbaar voor de goede verzorging van de proefdieren, de waarschuwingstelefoontjes en gewoon voor hun belangstelling voor de experimenten. Het personeel van de Tolakker rundveestal, waar ik cervix biopten heb verzameld dank ik ook voor de prettige samenwerking, het waarschuwen als de koeien gingen kalven en het verzamelen van de bloedmonsters.

Collega's en ex-collega's van de Verloskunde, later Afdeling Gezondheidszorg Herkauwers. Het was een leuke tijd. Bedankt voor de keren dat jullie voor mij insprongen als ik helemaal vastliep met experimenten en AZZ-diensten, ondanks het feit dat jullie zelf al zwaar belast waren met eigen diensten.

De rest van het ondersteunende personeel, de patiënten administratie, de mensen van het KI-lab en het biochemische lab, en nog vele anderen die ik misschien niet persoonlijk genoemd heb wil ik ook bedanken voor hun bijdrage aan en de getoonde belangstelling voor het proefschrift van deze verloskundige onderzoeker. De overige PhD's op de Afdeling Gezondheidszorg Herkauwers én Biochemie, Celbiologie en Histologie wil ik bovendien alle succes toewensen bij het afronden van hun promoties in de nabije of wat verdere toekomst.

Ik dank mijn familie en vrienden voor de steun en het begrip dat zij getoond hebben, vooral in het laatste afrondende jaar van het onderzoek. De vele verjaardagen en andere hoogtepunten, waar ik niet bij aanwezig kon zijn, vanwege diensten of nachtelijke experimenten of het vele schrijfwerk dat nog moest gebeuren hoop ik nu wat in te kunnen halen.

In het bijzonder bedank ik mijn ouders: Pa en Ma, jullie hebben het allemaal van een afstand gevolgd, maar toch hebben jullie de basis hiervoor gelegd. Dankzij jullie, heb ik het zelfvertrouwen gekregen om altijd voor het uiterste in mezelf te gaan. En hoe kon ik ook anders - met de naam die jullie mij gaven – dan kiezen voor de wetenschap.

Franklin, hoe hadden wij kunnen vermoeden, toen wij elkaar 19 jaar geleden ontmoeten op de AMS, dat wij 15 jaar later in Nederland elkaar het jawoord zouden geven? Het was en wordt een enerverend jaar voor ons: een promotie, een CD en als grootse hoogtepunt: ons eerste kind! Er is maar een manier waarop ik kan uitdrukken wat je voor mij betekent: "Franklin, je bent de

muziek van mijn leven". Daarom ben jij ook degene die, samen met Xannelou Mendeszoon mij zal bijstaan als mijn paranimfen, op de dag van de promotie. Een beter team kan ik mij niet wensen.

# Curriculum Vitae

Vidya Breeveld-Dwarkasing werd geboren op 13 maart 1966, te Paramaribo Suriname. Het VWO diploma werd behaald in 1985 aan de Algemene Middelbare School (AMS) te Paramaribo. In datzelfde jaar begon zij aan de Geneeskunde opleiding aan de Anton de Kom Universiteit te Paramaribo, na de eerste keer te zijn uitgeloot voor de studie Diergeneeskunde aan de Universiteit Utrecht. In 1986 maakte zij de oversteek naar Nederland om als parkeer studie Scheikunde te gaan studeren aan de Universiteit Utrecht. Na de vierde keer te zijn uitgeloot, werd zij in het najaar van 1988 alsnog toegelaten tot de studie Diergeneeskunde, die zij in 1996, als dierenarts met de differentiatie Landbouwhuisdieren, afrondde. In 1994, verrichtte zij na een grondige training in bedrijfsbegeleiding van de fertiliteit van melkvee te hebben gehad, een veldonderzoek naar aspecten die de fertiliteit van melkvee in Suriname bepalen. Dit onderzoek en de voorbereidingsperiode resulteerden in twee scripties die de fertiliteit van melkvee onder vochtige tropische omstandigheden beschrijven. Hiermee werden de eerste voorzichtige schreden op het wetenschappelijk pad gezet.

Na korte tijd als waarnemend dierenarts te hebben gewerkt, begon zij in 1997 als wetenschappelijk medewerker bij de Vakgroep Bedrijfsdiergeneeskunde en Voortplanting, later Hoofdafdeling Gezondheidszorg Landbouwhuisdieren. Daar werkte zij als junior docent/ dierenarts assistent in het verloskunde onderwijs en was de eerste promovendus die begon aan het onderzoek aan de cervix van het rund, binnen deze Hoofdafdeling. Dit onderzoek staat beschreven in het huidige proefschrift.

# List of Publications


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