Smooth muscle cells in bovine cervical ripening and dilatation

Contractility, degrading enzymes and inflammation

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Gladde spiercellen in cervix rijping en dilatatie bij de koe;
contractiliteit, proteolytische enzymen en ontsteking
(met een samenvatting in het Nederlands)

Proefschrift

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Want Godt de Heere is de Sonne ende schilt
Psalm 84, 12 (Deux-Aesbijbel)

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General introduction

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1.1 The uterine cervix in pregnancy and parturition

The uterine cervix has two very different functions during pregnancy and parturition. During pregnancy it is an important barrier that prevents the entry of bacteria into the uterine lumen, thus protecting the conceptus from becoming infected or expelled. To this end, the cervix is a rigid, tubular structure the lumen of which is sealed with a mucous plug. Insufficiency of cervical barrier function predisposes to intra uterine inflammatory processes resulting in death or preterm loss of a non-viable conceptus. Cervical insufficiency and uterine infection are common causes of preterm birth in women, which is a leading cause of infant mortality and postnatal neurological disorders [1-3]. By contrast, parturition is characterized by uterine contractions and cervical dilatation. The previously stiff cervical tissue becomes readily distensible and suited to dilation and allows passage of the fetus, forced by uterine contractions and abdominal straining. The rate and degree at which this softening and effacement occurs varies among species [4]. Despite differences in cervical anatomy between species, insufficient cervical dilatation is a cause of dystocia in many domestic animal species as well as in women, and a common reason for caesarean sections in both women and sheep. Since in cows, the cervix is relatively more muscular, fibrous and tightly closed during pregnancy than in the majority of domestic animal species, severe dystocia can result if it is not properly relaxed and dilated [5]. In cows, 17 % of all cases of dystocia are of cervical origin [6] and in beef cattle, 38 % of maternal cases of dystocia are due to incomplete cervical dilatation [7]. In view of the serious implications of cervical malfunction, knowledge of the regulation of cervical function and options for pharmacological intervention must, to date, be considered largely insufficient.

1.2 The anatomy of the cervix

In general, the female genital tract is histologically characterized by the presence of three different layers: A mucosal layer consisting of an epithelium and underlying stroma, a muscular layer, which in the bicornuate uterus consists of an inner circular and outer longitudinal layer and an outer serosal layer [8]. These three layers are also found in the cervix, in which the stromal layer is most prominent, and contains much fibrous tissue.

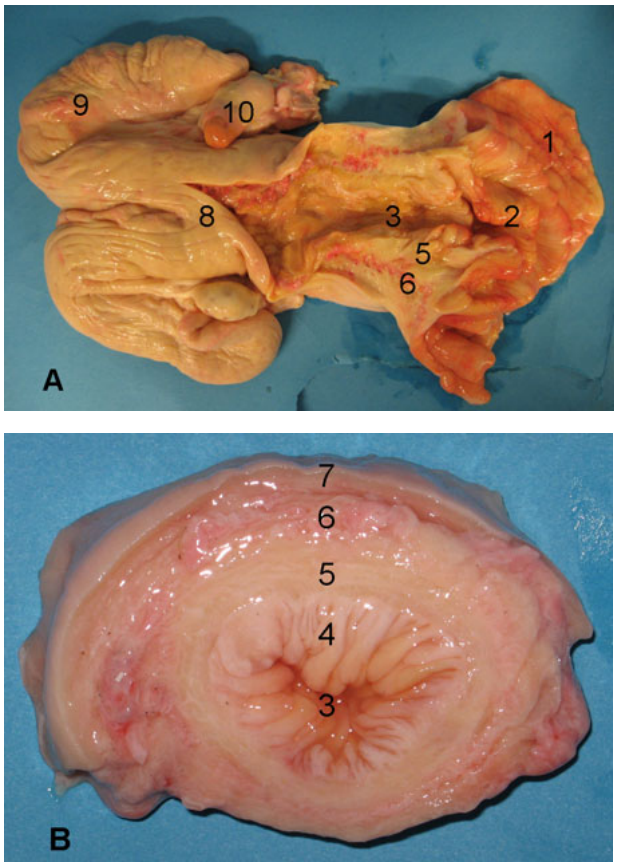

Figure 1.1 A) Dorsal view of the bovine genital tract with a longitudinal incision, to show the internal structures of the cervix. B) Cross sectional view of the bovine cervix. 1: Vaginal wall; 2: Caudal cervical rim; 3: Cervical lumen with protruding annular rings; 4: Epithelial and superficial stromal layer; 5: Deep stromal layer; 6: Outer muscular layer; 7: Serosa; 8: Uterine body; 9: Uterine horn; 10: Ovary.

The bovine cervix is a 6 to 13 cm long, firm and thick-walled tube with 3 to 4 annular projections of the wall that practically occlude the cervical lumen (Fig 1.1). The most caudal annular ring projects into the vagina as the “portio” with the external os as its centre. The cervical mucosa is further characterized by multiple branched longitudinal folds. Compared to rabbit, mouse and man, the cervix of cows has a relatively muscular nature [9-11] and within the organ, smooth muscle cells are distributed over the muscular and the stromal layers. Anatomically, the muscular layer is a caudal extension of the uterine myometrium and, like the uterus, consists of an outer longitudinal and an inner circular layer [12]. However, in the cervix these two muscular layers are not as clearly distinguishable as in the uterine myometrium, where they are well-developed and separated by a vascular layer [13]. The cervical stroma is composed of a thin superficial layer directly beneath the epithelium, and a thick deep stromal layer [14-16]. Macroscopically, the superficial stromal layer has a loose and pink appearance while the deep stromal layer is white and firm. Despite its fibrous macroscopic appearance, the deep stromal layer contains many smooth muscle cells, as has been shown with immuno-histochemical staining for smooth muscle actin (αSMA) [9,11]. The smooth muscle cells in the deep stromal layer are arranged in small bundles with a random orientation and separated by connective tissue, which contains blood vessels [17].

1.3 Cervical ripening: a two stage process

The cervix of the non-pregnant and early pregnant cow is firm, due to a high amount of extra cellular matrix (ECM) which consists of large amounts of types I, III or IV collagen, proteoglycans and a small amount of elastin. In the human cervix, collagen type I accounts for 80 % of the total cervical protein content [18,19] and in the cow the amount of collagen is approximately 35% and 70 % of the stromal dry weight respectively, for cycling and pregnant animals [20,21]. The collagen fibrils are reorganized before parturition and in preparation for degradation during parturition, to make the cervix easily distensible [22,23]. This cervical ripening process can be functionally divided into two stages; gradual ripening, which starts during

the last months of pregnancy, and final ripening which occurs as late as the onset of parturition [19,24,25].

The gradual cervical ripening process is an important prerequisite for a successful final ripening and cervical dilatation at parturition [26]. It is clinically characterized by palpable softening [27] and can objectively be determined with cervical compliance measurements [28-30] or fluorescence spectroscopy [31]. Gradual ripening is biochemically characterized by water imbibition, separation of collagen bundles and collagen denaturation [21,32]. The collagen denaturation at this stage may be due to the activity of cervical Matrix Metallo Proteinases (MMPs) [21], although this has not yet been investigated in a systematic fashion.

At final cervical ripening, which takes place just before and during cervical dilatation, collagen fibrils are denatured and digested by MMPs, as part of an “inflammatory” cascade [33-39]. Cervical tissue at this stage of ripening is characterized by a further decrease in collagen concentration and an increase in collagen denaturation [21] and water imbibition [40] which results in cervical tissue with an extremely soft, watery and well-distensible character. The cervical tissue is able to dilate, and to give way to pressure exerted by the fetal membranes or the fetus when the uterus contracts.

Cervical ripening and MMPs

Before the 1980’s, it was assumed that cervical ripening was caused by changes in the non- collagenous extracellular matrix and that collagen was not degraded at all [41,42]. Thereafter, it became apparent that collagen was degraded by MMPs during final cervical ripening [43-45]. MMPs are calcium and zinc dependent endopeptidases, which are secreted into the ECM as zymogen and subsequently activated by proteolytic cleavage. The MMP family is divided into five sub-groups, depending on their domain structures and their substrate specificities [43-45]. The gene expression for the individual MMPs is tightly regulated, which leads to a cell and tissue specific expression of the different MMP genes [46]. Once activated, MMPs are inhibited by two types of endogenous inhibitors: β2-macroglobulin and Tissue Inhibitors of Metallo Proteinases (TIMPs). TIMPs inhibit the activity of MMPs by formation of a stable complex and in fact, enzymatic activity depends on the balance between MMPs and TIMPS in the ECM [47]. Four

different MMPs appear to be involved in cervical ripening, i.e. MMP-1 (fibroblast collagenase), MMP-2 (gelatinase-A), MMP-8 (leukocyte collagenase) and MMP-9 (gelatinase-B) [24,48-51]. MMP-1, -2 and -8 cleave fibrillar collagen types I, II and III while MMP-9 cleaves collagen types IV and V and denatured collagen (gelatin). The latter is also cleaved by MMP-2. [43-45,52,53]. The differences in regulation and substrate specificity of the MMPs suggest that they might have different functions within the processes of cervical ripening. Some types of MMPs might already be active during late pregnancy and play a role in gradual cervical ripening, while other MMPs may be involved only in final cervical ripening.

Cervical ripening and cervical smooth muscle cells

In addition to their obvious contractile function, smooth muscle cells may play a major role in remodelling the ECM [54,55]. In the walls of both blood vessels and the vagina, smooth muscle cells are known to display either a muscular phenotype, characterized by the abundant presence of actin and myosin, or a secretory phenotype, characterized by the abundant presence of endoplasmatic reticulum and a large Golgi complex [56,57]. For the vascular smooth muscular cells, the presence of a secretory phenotype is coupled to vimentin expression [58]. Since smooth muscle cells in visceral organs rarely express vimentin [59], it is valid to ask whether stromal smooth muscle cells of the cervix do, because this would suggest that these cells have a secretory function and might play a role in gradual and/or final cervical ripening.

Final cervical ripening and inflammation

Final cervical ripening, collagenolysis and inflammation are closely related processes, as has convincingly been demonstrated by Junqueira et al. [60] who showed that during human parturition, invading granulocytes were surrounded by a halo of degraded collagen. The presence of chemokines, such as IL-8 is an important stimulator of granulocyte invasion. Cervical IL-8 concentrations increase with cervical dilatation [61-64], as is extensively documented in several species including man and rabbit [62-66], but not in the cow. Additionally, it has been reported that other pro-inflammatory cytokines, such as IL-1, TNFα and IL-6, are secreted within the cervix at parturition [35,61,67-69]. It has also been hypothesized by several authors

that a decrease in progesterone levels and leakage of cytokines or prostaglandins from the fetal membranes, leads to an increased expression of IL-8 and the onset of an inflammatory cascade within the cervix [33,34,70-72]. However, these hypotheses are mainly based on models of parturition induced by infectious agents or their derivates [37,73], and seem to ignore the existence of an endogenous gradual ripening process.

The inflammatory cascade leading to cervical ripening, as it is described in several species, is characterized by a similar series of events, such as leukocyte invasion and increased cytokine expression. This means that cervical ripening is closely orchestrated, and as well as pro-inflammatory cytokines, regulatory cytokines such as IL-10 may play a role in this process. To date, the contribution of inflammatory mediators other than IL-8 to cervical ripening is unclear, and the successive events within the inflammatory cascade have not been determined. It has however been suggested that the production of IL-8 by cervical smooth muscle cells is the first step of the cascade [73,74].

1.4 Cervical dilatation

During parturition, the previously firmly closed cervical lumen must dilate sufficiently to permit fetal expulsion. Dilatation of the cervix at parturition depends on two different phenomena, namely the process of cervical ripening and the onset of uterine contractions. The muscular layer (myometrium) of the uterus is relatively thick, and during pregnancy this layer even increases in size by hyperplasia and hypertrophy, enabling the uterus to perform powerful contractions during labor. Another factor that enables the myometrium to exhibit coordinated contractions during labor, is the abundant presence of gap junctions built up from connexin-43 proteins [75,76] in response to the pre-parturient decrease in plasma progesterone and increase in plasma estrogen concentrations [77-81].

During labour, uterine contractions force the ripened cervix to dilate [15,16]; actual uterine contractions are associated with simultaneous, temporary dilations of the ripened cervix [82,83]. After each contraction, the cervical diameter does not return to its previous size, but remains partially dilated. Progression of these processes results in complete cervical dilatation. However, in the cow the dilatation of the caudal cervix starts about 12

hours later than the increase in uterine contractile activity. This period may be necessary for completion of cervical ripening which is at least partially independent of the onset of uterine contractions [84]. Expulsion of the calf is accompanied by strong uterine contractions supported by abdominal straining efforts of the mother. Equally strong and even more frequent uterine contractions are present directly after fetal expulsion [85]. These contractions normally diminish within one day and likewise the cervical internal diameter decreases rapidly after parturition [86]. During the early post partum period, the cervical lumen must allow passage of placenta and lochiae, but it also has to contract to prevent invasion by bacteria, to reduce the size of the uterus and to prepare for a new pregnancy.

Cervical dilatation and cervical smooth muscle cells

It has been demonstrated in the parturient cow that the cervix dilates in response to uterine contractions. However, a detailed observation of changes in cervical diameter in combination with uterine electromyographic (EMG) measurements revealed that, occasionally, the cervical diameter temporally increased while at the same time the uterus did not contract [83]. This suggests that, at that moment the cervical diameter is influenced by contractions of the cervix itself. In fact, it has been shown extensively both *in vivo* and *in vitro,* in cows [87], sheep [88-90], rats [22] rabbits [29] and women [91-94] that the cervix exhibits muscular contractions. It is unknown whether these contractions arise from the muscular- and/or stromal layer, since, until now, cervical contractility of the outer muscular layer specifically has only been measured in parturient sheep [89]. In this latter study, it was noted that the EMG activity of the cervical outer muscular layer did not decrease at parturition, but it was not clear whether contractions of the outer muscle layer would cause an increase or a decrease in cervical diameter. The cervical outer muscle layer is a caudal extension of the uterine muscle layer, which might imply that these two tissues share the same function and mechanisms of regulation. Therefore, the question arises of how EMG activity of the cervical outer muscle layer develops in comparison to that of the uterus and how this relates to simultaneous changes in cervical diameter. In contrast, the stromal layer is not a caudal continuation of the uterine myometrium and the smooth muscle cells within this layer are organized in separate randomly oriented

bundles. This suggests that function and activity of the muscle cells in this layer may differ from those in the muscle layer. Until now, activity and function of the bundles of smooth muscle cells within the cervical stroma have hardly been investigated. One may well ask whether they show EMG activity, whether the bundle structure remains intact during cervical ripening and whether they show increases in connexin-43 expression during late pregnancy and at parturition, as in the myometrium [78], which would indicate improved coordination of activity between these cells.

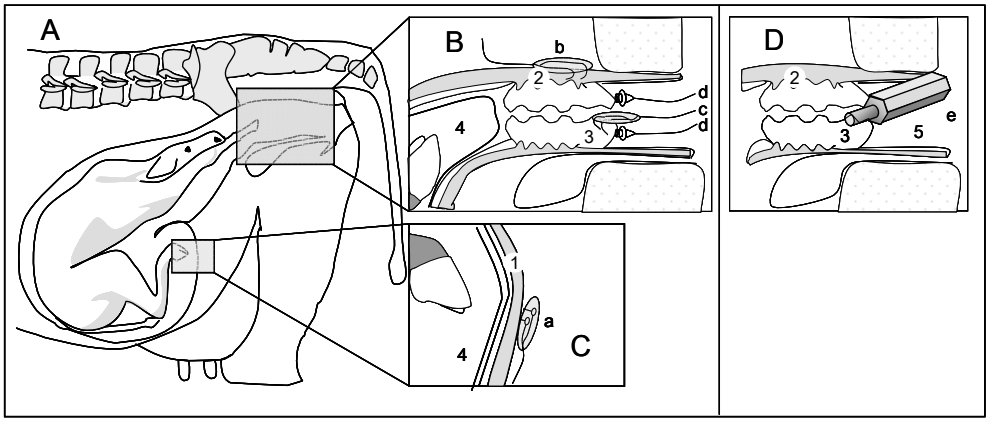

Figure 1.2 A schematic diagram of the pregnant uterus and cervix of the cow illustrating the location of the measuring devices. A: Overview of the genital tract, B: Detailed view of the cervical region with the EMG and ultrasound devices, C: Detailed view of the myometrium with the EMG electrode, D: Detailed view of the cervical region with the biopsy punch in position to take a sample of cervical tissue. 1: myometrium, 2: cervical outer muscle layer, 3: cervical deep stromal layer, 4: amniotic cavity with calf, 5: vaginal cavity. Instrumentation: a: bipolar uterine myometrial EMG electrode, b: bipolar abdominal EMG electrode in the cervical muscle layer, c: bipolar EMG electrode in the cervical stromal layer, d: transmitting and receiving cervical ultrasound transducer of the ultrasound cervimeter, e: biopsy punch.

1.5 The cow as a model for the study of cervical function

The cow is a suitable species to study the processes of cervical ripening and dilatation at pregnancy and parturition [17]. Previous studies from our laboratory have shown that a series of four biopsies are easily obtained from different spots of the caudal bovine cervix during the course of pregnancy and parturition, without causing serious side effects like abortion or dystocia [21]. Additionally, in this species, it is possible to perform abdominal surgical intervention at a late stage of pregnancy in order to place measuring devices onto the uterus and cervix [95]. Finally, the large size of the cervix enables researchers to distinguish between the different tissue layers within the cervix with regard to their biochemical changes, muscular activity and mRNA- and protein expression.

1.6 Objectives and Outline of the thesis

The general objective of this thesis was to improve our understanding of the functional involvement of cervical smooth muscle cells in cervical ripening and dilatation. As the cervical outer muscle layer is regarded as a continuation of the uterine myometrium, it seems reasonable to assume that a role in contraction would be the major function of this layer. Therefore, this layer was investigated with regard to its contractile activity using EMG measurements. The smooth muscle cells of the cervical stromal layer may have a contractile function and/or a function in the remodelling of the ECM; therefore this layer was investigated with regard to its contractile activity, its expression of smooth muscle actin and connexin-43, but also with respect to its vimentin expression and the activity of MMPs and inflammatory mediators.

The following questions were addressed in this thesis:

1. Do the outer smooth muscle layer and stromal layer of the cervix exhibit contractile activity during late pregnancy, parturition or shortly after parturition and, is this activity in one way or another related to myometrial contractile activity and changes in cervical diameter?

2. Are the smooth muscle bundles of the cervical stromal layer prepared for contraction at parturition, or do these cells predominantly have a secretory function?

3. Which MMPs are involved in the gradual stage of the cervical ripening process in the cow, and which ones in the final stage?

4. Which pro-inflammatory and regulatory cytokines are involved in the inflammatory cascade leading to cervical ripening in non-complicated parturition at term in the cow, and is their presence related to the invasion of leukocytes?

In general, two approaches were used to study the bovine cervix. The first was to implant electrodes onto the cervical stromal and outer muscle layer and, onto the uterine myometrium in late pregnant Holstein Friesian cows, to determine the EMG activity (Fig. 1.2B and C). In the same animals ultrasound transducers were sutured onto the caudal cervical rim to measure changes in cervical diameter [83] (Fig 1.2B). After a recovery period, parturition was induced with prostaglandin F2α, and the EMG activities and cervical diameter were measured almost continuously until two days after delivery of the calf. The second approach was to obtain biopsies from the bovine caudal cervical rim in the same animal repeatedly. These biopsies were taken at 185 days of pregnancy, at 275 days of pregnancy (term), just after spontaneous calving and at 30 days after calving (Fig 1.2D). They were analyzed by means of fluorescence immuno histochemistry, histochemistry and Western blot techniques, enzyme activity assays, ELISA and RT-PCR techniques.

Outline of the Thesis

Chapter 2 and 3 describe the EMG activity of the cervical outer muscle layer and cervical stromal layer before, during and after parturition in the cow. To this end, EMG electrodes were attached on the cervical outer muscle layer and sutured onto the myometrium of 8- month pregnant cows by abdominal surgery. Cervical stromal EMG electrodes and ultrasound crystals for measurement of the cervical diameter, were attached to the caudal cervix via a vaginal approach. After induction of calving with a single i.m. injection of PGF2α, the EMG activities and cervical diameter were

almost continuously measured until two days after expulsion of the calf. The EMG activity of the cervical tissue was analyzed with regard to the amount of coordinated activity, the amplitudes of the EMG activity and duration of EMG activity. These were compared with similar parameters for EMG activity of the uterine myometrium and with simultaneous changes in cervical diameter.

Chapter 4 describes the smooth muscle bundles in the cervical stroma with regard to their morphological appearance at days 185 and 275 of pregnancy, at parturition and at 30 days after parturition. The expression of smooth muscle actin, the intermediate filaments vimentin and desmin, and the gap junction protein connexin-43 by smooth muscle cells was semi-quantitatively assessed at these time points using RT-PCR and immunohistochemical staining.

Chapter 5 reports on the semi-quantitative assessment of mRNA and protein expression for MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in serial cervical biopsies which were recovered from the same cow at days 185 and 275 of pregnancy, at spontaneous parturition and at 30 days after calving. Messenger RNA expression was determined using RT-PCR, while the protein levels of the MMPs and TIMPs were determined using zymography, Western blot techniques and ELISA. Additionally, the localization of MMP-2 was determined using immunohistochemistry.

In Chapter 6 the mRNA expression patterns of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα were assessed, together with the regulatory cytokine IL-10, in cervical tissues recovered at days 185 and 275 of pregnancy, at parturition and at 30 days after calving. Furthermore, the numbers of neutrophils and eosinophils were determined at each of these sampling times.

Finally, in **Chapter 7,** general conclusions are drawn from the different experiments, and implications and suggestions for further research are proposed.

A summary of the outline is given in Figure 1.3.

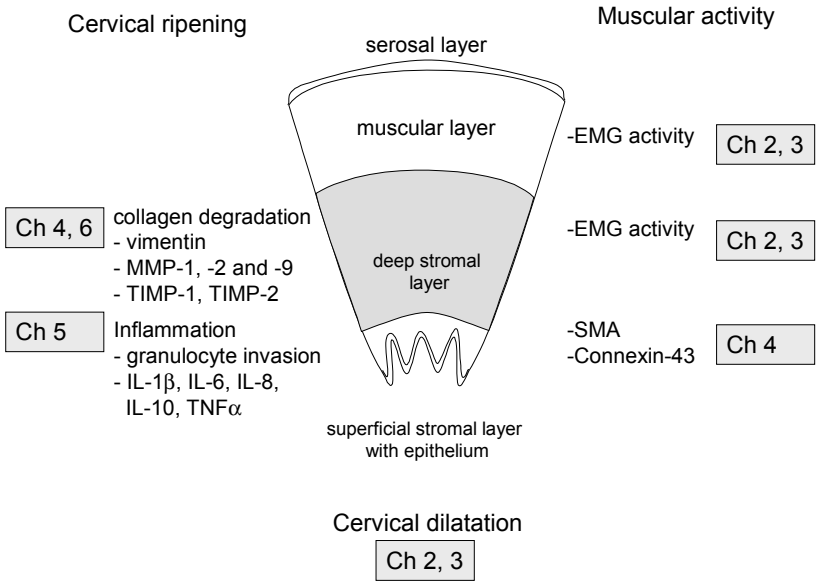

Figure 1.3 Outline of the thesis based on both possible functions of the smooth muscle cells within the cervical deep stromal layer and outer muscular layer. The shaded boxes indicate the chapters in which the topics are addressed.

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7

EMG activity of the muscular and stromal layer of the cervix in relation to EMG activity of the myometrium and cervical dilatation in PGF2α induced parturition in the cow

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Abstract

The goal of this study was to quantify and characterize the EMG activities in the cervical outer muscular layer and in the cervical stromal layer, and to characterize their relationship with myometrial EMG activity and cervical dilatation during PGF2α-induced parturition in term pregnant cows.

We continuously measured the EMG-activity of the uterine myometrium and cervical outer muscular layer as well as the cervical stromal layer in five cows using bipolar electrodes while at the same time measuring changes in the cervical diameter with ultrasound cervimetry. This was done from the moment a prostaglandin-analog was injected until the expulsion of the calf.

In contrast to the cervical stromal layer, the cervical outer muscular layer showed distinct EMG activity, which began to increase at about the same time as the EMG activity of the myometrium, i.e. some12 h before the start of cervical dilatation. However, the rate of this increase was lower than in the myometrium and it was not characterized, like in the myometrium, by an increase in maximum EMG amplitude. Although the cervical outer muscular layer showed contracture and contraction like EMG activity in unison with in the myometrium, it was also characterized by a more irregular EMG activity, which occurred independently from the myometrium.

These data suggest that while the outer muscular layer of the cervix may be considered to be a caudal continuation of the myometrium, it also displays activity independently from the myometrium. The physiological relevance of this activity remains to be explored.

Introduction

It has been established that cervical dilatation, whether at term or preterm, is the result of mechanisms that are very similar to an inflammatory reaction [1-3]. The resultant disruption of the extra cellular matrix softens the cervix and uterine contractions then actively cause funneling, shortening and subsequently dilatation of the cervix. The temporal relationship between the uterine contractile activity and the opening of the cervix during parturition has recently been described in cows from initiation of the parturition until vaginal expulsion of the fetus [4,5]. The uterine myometrial (MYO) electromyographic (EMG) activity started to increase at about 15 h before the onset of dilatation of the caudal cervix. In addition to this relationship between MYO EMG activity and cervical dilatation, it has been observed that in the parturient cow the cervical diameter momentarily increased and decreased in the absence of uterine contractions [4-6]. Similar observations have been made in humans [7]. This suggests that the cervical diameter is influenced by smooth muscular tissue from within the cervix.

Two other observations also suggest a role for the smooth muscle cells in regulating the cervical diameter. The smooth muscle content of the cervix is large and for example in the human cervix can reach up to levels as high as 40 or 45 % [8], which may even increase during the course of pregnancy [9]. Further, cervical smooth muscle shows contractile activity, as demonstrated by EMG recordings in parturient woman [10-12] as well as by organ-bath studies with strips of human, rabbit, rat and bovine cervical tissue [13-16].

Smooth muscle cells in the cervix are distributed in two layers, an outer cervical muscular layer (CML), and a deep cervical stromal layer (CSL). The latter consists of dense connective tissue with a considerable amount of smooth muscle cells dispersed within it [17]. The smooth muscle cells in these two layers are differently organized. The smooth muscle cells in the CSL form small bundles, which are scattered through the extracellular matrix, but in the CML, they are more or less orientated in an inner circular and an outer longitudinal layer (personal observation from immunohistochemical studies). The smooth muscle cells in the CSL are not clearly

connected with the MYO, but the CML is morphologically regarded as a caudal continuation of the MYO [18].

Although highly neglected until now, these differences in the organization of smooth muscle cells in the two layers could imply a different functionality during cervical dilatation. If this notion was true, one might expect that the two layers would show different levels of contractile activity during parturition, which would result in different EMG activity patterns. We were therefore compelled in this study, to evaluate the activity of the CSL and the CML separately in relation to cervical dilatation.

The question, we posed to ourselves was, if any EMG signal could be registered from the CSL in vivo. We supposed that the CML, being a continuation of the MYO, would be subject to the same regulatory mechanisms as the MYO and its EMG activity would therefore be more or less in concert with the MYO contractility patterns. If the latter was the case, it would contradict results of in vitro contraction studies with cervical and uterine tissues, and nitric oxide synthase (NOS) measurements in rats, that indicate that the cervix relaxes rather than becomes contractile during parturition [19,20]

To characterize the activity and explore possible functions of the smooth muscle cells in the CSL and CML, we recorded and quantified EMG signals of both layers separately and established their temporal relationships with changes in the cervical diameter and myometrial EMG activity before and during PGF2α induced parturition in cows.

Materials and methods

Animals and Surgery

Five pluriparous pregnant Holstein Friesian cows were used in this experiment. They were housed in individual stands from the 6th or 7th month of pregnancy onwards until the start of the measurements. They had a normal daylight regime and a food regimen appropriate to their pregnancy state. The Animal Experiments Committee of the faculty of Veterinary Medicine, Utrecht University approved the protocol used.

Surgery was performed at least 10 days before calving was induced on day 274, after a 48 h fast and 24 h water deprivation. We used the protocol previously described by us [5] with some adaptations. In short, two bipolar AgAgCl electrodes were sutured on the surface of the MYO of the pregnant uterine horn. In addition, a fishhook-shaped stainless steel bipolar electrode was attached on the central dorsal surface of the CML. This electrode was prepared from two fishhooks (Partridge of Redditch, UK), that were connected to a wire and imbedded in epoxy (Bison kombi snel®-rapide) parallel to each other with 5 mm distance in between. This was covered with silicone, leaving the tips of the hooks uncovered.

In two cows, the uterus and cervix were approached by mid-ventral laparotomy under general anesthesia, with the cows in dorsal recumbency. Subsequently, the left circumflex ilial artery was catheterized, after the cows had been turned to right lateral recumbency. Because, it appeared to be difficult to reach the cervix with this approach, in the remaining 3 cows the uterus and cervix were approached by laparotomy of the left flank using a paravertebral block anesthesia with the cow standing. After that, the catheter was placed in the left circumflex ilial artery under general anesthesia as in the other 2 cows. The catheter and the wires from the electrodes were tunneled subcutaneously to the dorsal sublumbar area. The ends were exteriorized, wrapped in an alcohol soaked gauze and stored in a plastic bag. Postsurgical treatment involved a non-steroidal anti-inflammatory agent, Flunixine meglumide (Bedozane ®) iv for 2 days and an antibiotic treatment with Ampicillin (Praxavet Ampi®) im for 5 days. After a recovery period of 2 days the cows were placed in an individual pen (1.25 by 2.50 m) with ad libitum food and water. Here they stayed until the end of the experiments.

Experimental protocol

The experimental protocol started at day 270. Between d 270 and d 274 of gestation, blood samples were collected daily around 8.00 am and 5.00 pm. After d 274, blood samples were taken every 4 hours. The blood was collected in heparinized tubes and centrifuged (3000 rpm, 7 min). The plasma was removed and stored at –20°C until analysis.

Before induction of the parturition, the EMG activity of the CML and the MYO were recorded daily for at least 2 to 3 days during 4 h periods, to characterize the basal pregnant EMG activity at term and to verify that parturition had not already started before on the time that the PGF2α injection had been scheduled.

At d 274, the cows underwent additional instrumentation immediately after a 4 h control recording. This additional instrumentation involved suturing of two ultrasound crystals on the caudal cervical rim as described earlier [5] and the attachment of a bipolar stainless steel electrode (similar as in the CML) in the caudal dorsal CSL. Here we approached the CSL from the luminal side. After this, EMG activity of the MYO, CML and CSL, as well as the change in the cervical diameter were continuously registered. This was done during an additional 4 h period, before the cows received a PGF2α analogue (Prosolvin ®, Intervet Boxmeer, The Netherlands, im, 2 ml). After the injection, all recordings were continued until the start of the expulsion.

The signals from EMG electrodes were amplified (model 11-4123-01; Gould Inc., Cleveland, OH or model UME-47; Schwarzer, München, Germany) and analog band pass filtered from 0.05 up to more than 10 Hz. The signals from the ultrasound transducers went to a cervimeter [4]. All signals were digitized by a multichannel analog/digital converter (National Instruments, Austin, Texas, USA), sampled with 40 Hz and stored using Labview 5.0 (National Instruments, Austin, USA).

To prevent that any harm would come to the animals or that damage occurred to the instruments by electrodes become trapped between fetal parts and the walls of the cervix or vagina, we removed the ultrasound crystals and the stromal cervical EMG electrode when the head of the calf protruded into the vagina. It was assumed that, at that time the cervix had already been completely dilated.

Data analysis and Statistics

Serum progesterone levels

Serum progesterone levels were measured by a validated direct solid phase ^{125}I radio immuno assay (RIA) as previously described [21].

Statistical analysis of the hormone concentrations was performed in SPSS with a repeated measures test.

Cervical dilatation

The data obtained from the cervimeter data were averaged over consecutive 10 sec periods. Following this, a bilinear non-linear regression (NLR) analysis was performed in SPSS (version 12.0.1,Chicago, USA, 2003), as has been described previously [4,5]. With this NLR model (Fig. 2.1) we identified the transition point (t1), which is the time point (h) when the cervix started to dilate rapidly. Additionally we calculated the rate of increase of the cervical diameter (s1 (cm/h)) before the transition point, the diameter of the cervix (cm) at the transition point (y1) and the rate of increase of the cervical diameter (s2, cm/h) after the transition point. For reason of clarity, s1 is indicated only in Fig. 2.1C and not in Fig. 2.1A.

The following formula was used.

if time <T1 predicted value = s1* time + y1.

if time ≥ T1 predicted value = s2 * (time - t1) + y1.

After obtaining these parameters for the individual cows, overall means (±sd, n=5) were calculated.

Myometrial and cervical EMG activity

The raw data obtained from the EMG recordings were qualitatively analyzed. To this purpose, we used the following definitions: A spike is a bipolar change in voltage not exceeding 2 seconds. A burst is a group of 3 or more spikes with a frequency of at least 0.5 Hz. A contracture is a period of EMG activity lasting longer than 4 min which is build up out of at least 30 spikes or 15 bursts, the interval between separate spikes or bursts never exceeding 30 sec. When a period of activity was build up out of spikes or bursts of low frequency and short duration, they were regarded as being irregular.

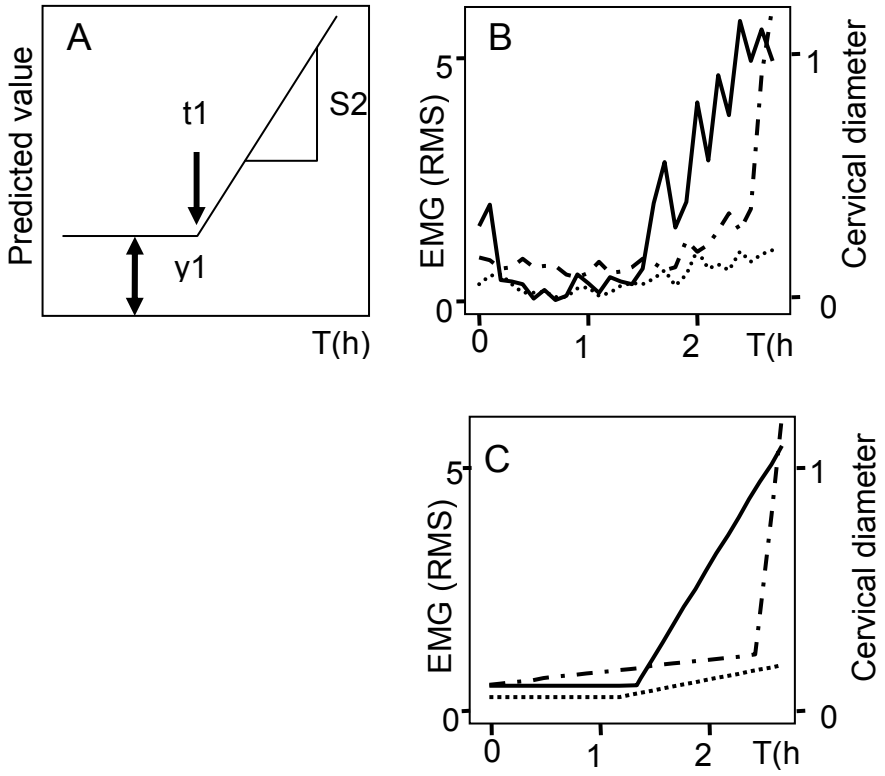

Figure 2.1 A) Schematic representation of how the NLR parameters were derived in each cow. Time after injection = time after injection with PGF2α analogue; Predicted value = either cervical diameter (cm) or uterine EMG activity (RMS, mV); y1 = basal initial value; t1 = transition point when the value starts to increase; s2 = rate of increase in cm/h or RMS/h respectively. B) Typical example of the mean cervical dilatation per hour (cm) (· – · – ·) and the mean RMS per h of the cervical muscular layer (·····) and myometrium (——). C) The predicted means of the same data shown in B, after NLR analysis. (B and C are obtained from the same cow.)

Record was made of the following events: 1) The occurrence of any type of EMG activity in the MYO, the CSL or the CML; 2) the occurrence of any sort of temporal relationship between the EMG activity of the MYO, the CSL or the CML; 3) any type of correlation between specific EMG activity patterns and the stage of cervical dilatation.

Before the EMG data were quantitatively analyzed, they were digitally high pass filtered (0.125 Hz, Butterworth). Subsequently, root mean square values (RMS) were calculated for each consecutive 10 sec period. Due to the presence of noise, the RMS values appeared to have a certain basal level. This basal level was subtracted from the RMS value before the data were further analysed. Extremely high RMS values of EMG signals were compared with the original EMG recordings and discarded, when they appeared to be obvious artefacts.

Finally, NLR analysis of the EMG recordings was performed using the following formula:

if time < T1 predicted value = y1.

if time ≥ T1 predicted value = s2 * (time - t1) + y1.

We identified the transition point t1, which is the time-point (h) when the EMG activity started to increase; y1which is the mean RMS value (mV) of the EMG signals during the stage before the transition point and s2, the rate of increase of EMG activity (mV/h) after the transition point had been reached. We did not calculate the slope of the EMG activity before the transition point because the uterus appeared to be almost completely silent during that time which is in accordance with an earlier study [22].

In addition, the increase of EMG activity (RMS) relative to the level of EMG activity before the transition point was calculated (s2/y1*100=%/h). All parameters were first obtained for individual cows, after which the overall means (± sd, n = 5) were assessed.

By definition, the RMS value of an EMG signal during a certain period depends on the amplitude, duration and frequency of the occurring spikes. Consequently, an increase in RMS value over this period may be caused by an increase of a single one of these variables or by a combination of

both. Therefore, in addition to the mean RMS, we analyzed the recordings for changes in these variables.

Quantification of the maximal EMG amplitudes in MYO and CML

The amplitude of the EMG signal is one of the factors contributing to the RMS values. An increase in the amplitude of the spikes from an EMG signal is a sign of increased uterine synchronization such as occurs when parturition progresses [23,24] as well as around estrus [25]. Therefore, the maximal amplitude of the EMG signal was determined for consecutive 15 min periods. This was done for the complete recordings from PG-injection to the moment that maximal cervical dilatation had been reached. For practical reasons, only positive values were determined. For each individual cow, these data were analyzed with NLR as described above, to identify sudden changes in the maximal EMG amplitudes in relation to the time after PGF-injection.

Quantification of activity time in MYO and CML

The amount of time that electrical activity occurs within a certain time unit is another factor that contributes to the RMS values of EMG activity. We calculated the amount of time that EMG activity occurred per hour, as previously described by Bajcsy et al. [26]. In short, RMS values per 1 sec period were calculated. The RMS values were considered to express true EMG activity when they exceeded twice the mean RMS value of the complete recording from PG injection until calving. All these 1s periods in which EMG activity occurred were summed for each consecutive hour. We called this parameter activity time (AT (min/h)). For each cow, these values of AT were analyzed with NLR as described above, to assess if any sudden and significant change in the AT occurred at a certain time-point during the period of recording.

Results

EMG activity patterns in the MYO and CML before induction of parturition

During late pregnancy and before induction of calving, both uterus and CML displayed contractures. The duration of these contractures in the MYO (12.1 (± 2.0) min) was not significant different of that in the CML (11.6 (± 2.1) min). However, the frequency of these contractures was higher in

the MYO (13.8 (± 2.4) per day) than in the CML (9.8 (± 3.7) per day) ($P<0.05$, paired samples T-Test). When a contracture occurred in the CML, it was almost always simultaneously with a contracture in the MYO. A difference between MYO and CML was that a contracture of the MYO was built up of bursts of 3-7 spikes, while a contracture of the CML was build up of bursts that varied in number of spikes, or single spikes that were not grouped together as a burst. Another difference was, that the CML, in contrast to the MYO, showed EMG-activity in between the contractures, as Fig. 2.2A shows. This irregular activity could vary from 1-2 isolated spikes/min to 1-2 bursts /min.

Outcomes of calvings and hormone analysis

All 5 cows had a normal spontaneous vaginal delivery of a single calf in anterior presentation. The calves were born at 40.6 (± 7.6) h after the PG injection, which on average was 0.6 (± 0.3) h after removal of the ultrasound crystals and the CSL EMG electrodes from the caudal cervix. Before the PG injection, the mean plasma progesterone levels were 4.8 (± 0.8) ng /ml. At 4 h after PG injection these levels had declined significantly to 2.0 (± 0.3) ng/ml ($p< 0.002$). From this point onwards, they gradually decreased to a level of 1.1 (± 0.3) ng/ml at the time of expulsion.

Cervical dilatation

The mean cervical diameter, before the time that it started to dilate, ranged from 1.8 to 2.9 cm between the 5 cows, with a mean of 2.4 cm. The caudal cervix started to progressively dilate at 28.6 (± 4.0) h after PG-injection with a rate of 3.8 (± 1.4) cm/h. The time interval between the onset of the cervical dilatation and the moment that maximal dilatation had been reached was 4.9 (± 2.1) h.

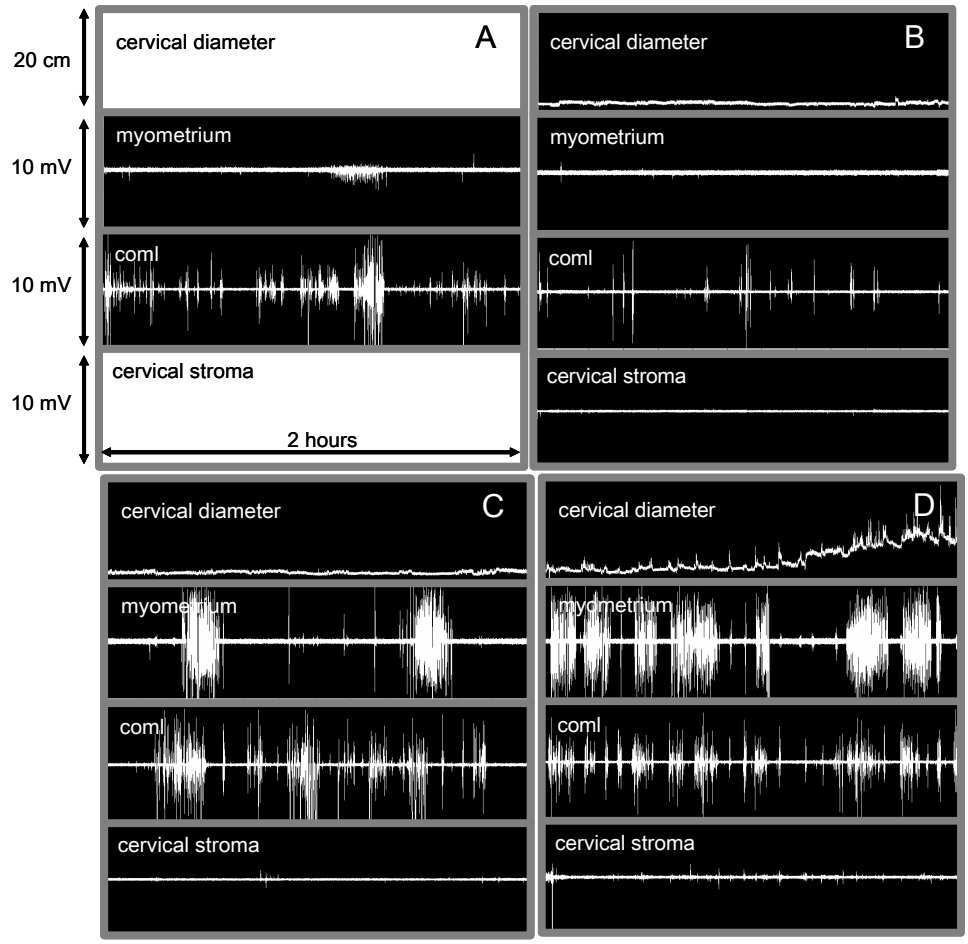

Figure 2.2 Representative examples of 2 h recordings (from the same cow) of the cervical diameter and EMG activity at 4 different stages between term pregnancy and fetal expulsion. A) At 8 hours before PG-injection on day 274 of gestation (term pregnant). B) The period before the start of cervical dilatation, when the MYO is quiescent. C) The period before the start of cervical dilatation, when the MYO EMG activity is steadily increasing. D) The period during which both cervical diameter and MYO EMG activity increases. Notice that in the pre-induction recordings (A), cervical diameter and EMG activity of the CSL were not recorded and also that CSL EMG activity is very weak to almost non-existent. MYO: myometrium; CML: cervical muscular layer; CSL: cervical stromal layer.

Quantitative and qualitative changes of EMG activity

As can be seen in Fig. 2.1, where for one cow the mean RMS value per hour have been potted (Fig. 2.1B), the graph which was obtained after NLR analysis (Fig. 2.1C) looks very similar. In fact, the transition points which were calculated by NLR approach could already be visually identified in Fig 2.1B. The transition point that marks the beginning of the increase in EMG activity of the MYO and the CML occurred earlier than the transition point of the cervical dilatation (see Fig. 2.3). This was true in all cows. The interval between the increase of EMG activity and cervical dilatation was 12.5 (± 3.8) h for the MYO and 12.1 (± 3.9) h for the CML. The EMG activity in the CSL was very low (Fig. 2.4A) and it was difficult to distinguish an EMG signal that extended above the noise level. Therefore, it was not possible to analyze these recordings similar to the MYO and CML.

The mean RMS value during the phase before the transition point of EMG activity was 0.53 (± 0.18) mV for the MYO and 0.65 (± 0.12) mV for the CML (Fig. 2.4A). During this period of low activity, in 2 cows, neither CML nor MYO showed any contracture activity, but the irregular activity of the CML remained (Fig. 2.2B). In the other 3 cows, the contracture activity remained in both MYO and CML but with relative low amplitude. The contractures occurred in most cases simultaneously in both locations.

After the transition point, the EMG activity of the MYO increased significantly faster (27.4 (± 17) %/h) than that of the CML (5.8 (± 3.8) %/h, $P< 0.05$, paired samples T-test).

During this period of increased EMG activity, the MYO and CML first displayed contractures. But within the contractures, bursts tended to aggregate, and pauses between separate groups gradually increased. MYO EMG activity changed until it finally showed 10 to 30 min periods of continuous EMG activity interspersed by mainly 2-3 min periods of inactivity. During the period of EMG activity, all bursts had nearly the same maximal amplitude, a duration of about 10 sec and were separated by 15-sec intervals. In contrast, the CML showed bursts of different lengths randomly mixed with spikes. The CML showed 2-3 min periods of inactivity simultaneously with the MYO.

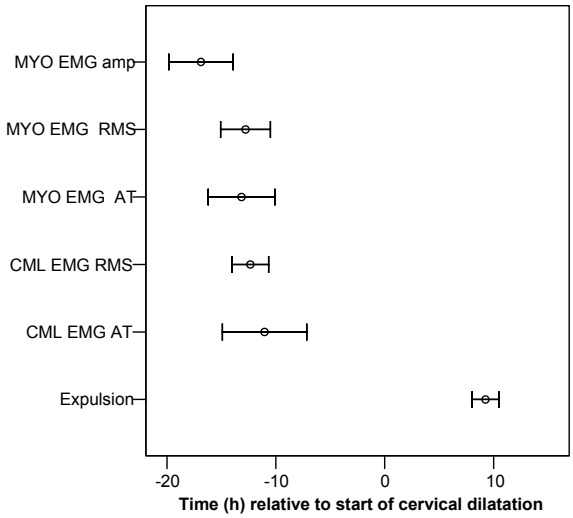

Figure 2.3 The times (n=5, mean ± sem) at which the calculated values of the EMG activities started to increase, relative to the start of cervical dilatation (t=0). MYO: myometrium; CML: cervical muscular layer; EMG amp: EMG maximal amplitudes per 15 min period; EMG RMS: EMG activity based on RMS values; EMG AT: EMG activity based on the occurrence of EMG activity per hour; Expulsion: the moment that the expulsion of the calf finished. In the case of CML, no transition point for the EMG amp could be identified.

Quantification of the maximal EMG amplitudes in MYO and CML

To determine whether the increase in RMS values of the MYO and CML was caused by an increase in maximal EMG amplitude, an increase in activity time (AT) or by a combination of both, these parameters were separately plotted in a graph (Fig 2.4B) and subjected to NLR analysis. Already at 16.6 (±7.1) h. before the cervix started to dilate (Fig. 2.3 and 2.4B) the maximal EMG amplitude of the MYO increased steadily with a rate of 8.4 (± 4.5) %/h relative to the initial value of 0.64 (±0.29) mV. The maximal EMG amplitude of the MYO increased significantly earlier than the RMS values ($p<0.05$, paired samples T-test). In the CML, no transition point could be calculated for the maximal amplitudes as was done for the MYO (Fig. 2.4B). The mean maximal amplitude of the CML between the 5 cows over the complete period between PG injection and onset of the expulsive phase was 1.2 (±0.2) mV.

Quantification of activity time in MYO and CML

Before the transition point, the AT was 2.0 (± 0.5) min/h in the MYO and 4.2 (±1.8) min/h. in the CML. The AT increased before the cervix started to dilate. In the MYO, this time interval was 12.9 h (± 4.1 h) and in the CML it was 10.8 h (± 5.0 h) (Fig. 2.3), the difference in interval being not statistically significant (paired samples T-test). In addition, they did not differ with regard to the interval between the onset of increase in RMS values and cervical dilatation. In the MYO, the AT increased with 0.65 (± 0.35) min/h, and in the CML with 0.50 (± 0.40) min/h (Fig. 2.4C).

Discussion

This study is the first to separately quantify and describe in detail the EMG-activity of the two smooth muscle containing layers of the cervix, the CML and CSL, and to relate it to the EMG activity of the uterus (MYO) and the start of cervical dilatation.

In this study, in the term pregnant cow, not only the MYO but also the CML showed EMG activity. This activity could be characterized as contractures. Mean frequency and length of these contractures were the same at these two locations, and corresponded accurately with previously published values in cows [27]. However, in contrast to the MYO, the CML also displayed distinct irregular EMG activity between the contractures. Similar observations have been made in recordings of cervical EMG activity in sheep [28] and human [10,11,29]. This suggests that the smooth muscle cells of the CML and MYO are differently regulated, or that these two locations of the genital tract are driven by their own pacemaker cells.

In contrast to what might be expected from reports in which an increase in cervical NOS expression and NO levels during labor was demonstrated [19,20], the EMG activity in the CML did not decrease before dilatation of the cervix. Instead, we saw a clear increase of contractile activity, which is

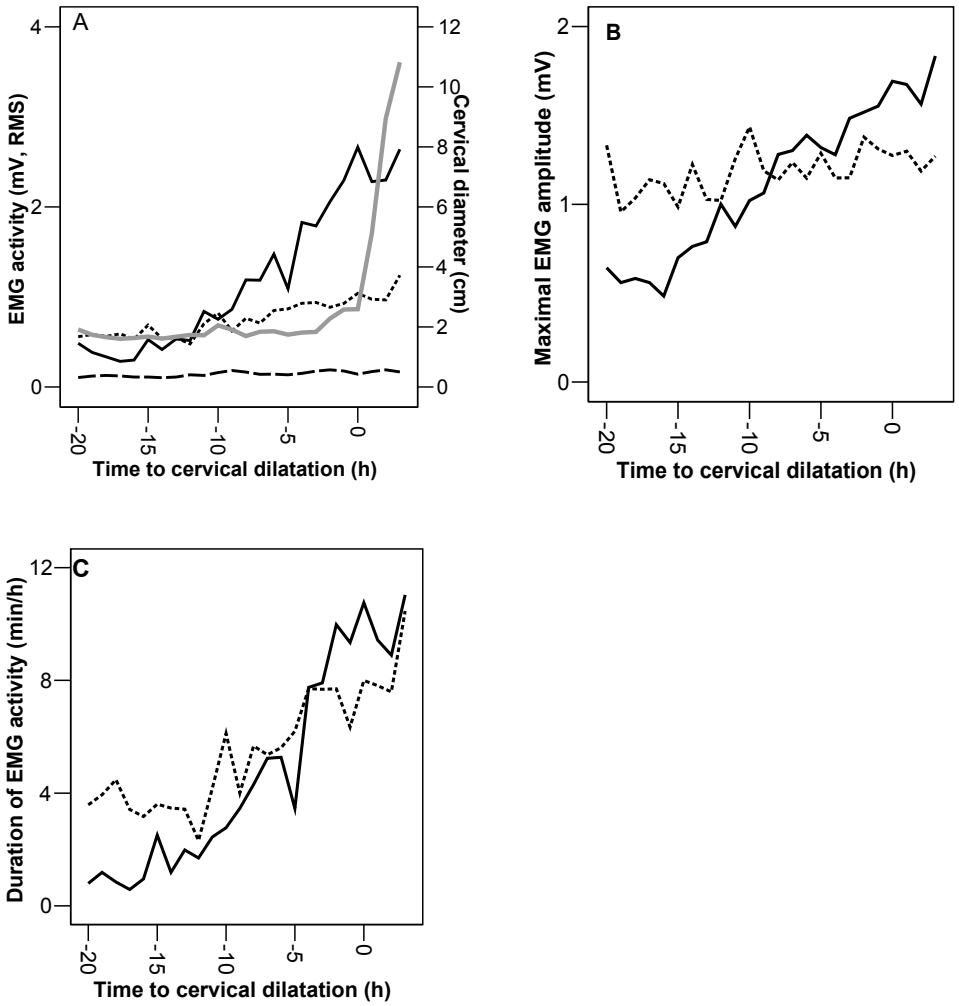

Figure 2.4 (A) The course of EMG activity of the myometrium, cervical muscular layer and cervical stromal layer and the change of cervical diameter relative to the start of cervical dilatation (t=0) means per hour (n=5). A) The course of the RMS values of the EMG activity and the cervical diameter. B) The course of the mean maximal EMG amplitude per 15 minutes. C) The course of the mean activity time (AT) of the EMG activity. (——): cervical diameter; (——): myometrial EMG activity; (----): cervical muscular layer EMG activity; (— —): cervical stromal layer EMG activity

in agreement with qualitative descriptions of the cervical EMG activity in parturient sheep [28]. The observation that the CML and MYO EMG activity started to increase at nearly the same moment, and the fact that their EMG activity pattern changed during the same period before cervical dilatation, could lead us to conclude that the CML is to be considered as a caudal continuation of the MYO also from a functional point of view.

However, when we analyzed the data in more detail, we also noticed some differences between the MYO and CML. Firstly, during cervical dilatation, the pattern of EMG activity in the CML was less organized than in the MYO. Secondly, the rate of increase of EMG activity in the CML was smaller than in the MYO. These two observations might lead to the speculation that around cervical dilatation the increase of CML contractile activity is dampened, compared to the MYO, for example by an enhanced local production of NO in the CML. However, recent data from our group indicate, that the expression of inducible NOS within the stroma of the caudal bovine cervix is decreased at calving [30]. Yet it could be that the iNOS activity in the CML differs from that in the cervical stroma and is stimulated during calving. If this appears true, the contractile activity of the CML could still be dampened by NO. It would also be in agreement with studies in both women and rats, which report high cervical iNOS concentrations during parturition [31-34] and that NO inhibits contractions of the human cervix in vitro [35]. Another explanation for the lower increase rate and less organized EMG activity, could be that local stretch of the CML, caused by a funneling like process such as occurs in laboring women [36] results in different conducting properties of the smooth muscular cells. A possible effect could be the release of locally produced agents, such as prostaglandins which may also dampen the contractions, as is shown by several in-vitro contraction studies [14,16,37].

The difference between the MYO and CML in the increase in the RMS values of the EMG activity can at least be partly explained by a difference in EMG amplitudes between the two sites. In the MYO, the increased EMG activity around cervical dilatation was preceded by and coincided with increased maximal EMG amplitudes. This increase has also been reported by others [38,39] and may reflect an increased synchronization of the

depolarisation of smooth muscle cells around the MYO electrode, a process that may be less developed in the CML. Gap junctions play an important role to enable such synchronization [40,41]. Therefore, it would be interesting to take repeated biopsies from the MYO and the CML, to investigate if the two sites differ with regard to the numbers of gap junction proteins during parturition.

The observation that in the MYO maximal EMG amplitudes began to increase earlier than the total EMG activity (Fig. 2.3) implies that synchronization of the smooth muscle cells by gap junction formation occurs relatively early in the parturition process, while the increase in AT possibly due to circulating or locally produced uterotonins follow at a later stage.

Not only with regard to maximal EMG amplitudes, but also with regard to the AT, there were differences between MYO and CML. During late pregnancy, the AT in the CML was about twice that of the MYO, probably caused by the occurrence of irregular EMG activity in between contractures in the CML. At the onset of cervical dilatation however, the AT's were almost at the same level at both locations. A relatively more steep rise in AT in the MYO than in the CML appears to be responsible for this effect. During calving also the myometrium itself shows contractions at higher frequency in the uterine horns than in the uterine body that is connected with the cervix [42]. This is thought to create a pressure gradient that assists the movement of the uterine content in a caudal direction, towards the cervix and pelvic canal. In vivo studies with human myometrium and cervix also demonstrate differences in properties between the MYO and cervical tissues both with respect to spontaneous contractile activity as to their responsiveness to different agents like NO and prostaglandins [16,37,43,44].

We observed no appreciable EMG activity of the CSL with the current experimental settings. This is in contrast to what we expected, based on the abundant presence of smooth muscle cells, as proven by immunohistochemical staining for smooth muscle actin-α [30] and our yet unpublished observation that tissue strips of the CSL display a distinct contractile response to oxytocin. Moreover, it is seemingly in contrast with

findings in women where EMG signals have been recorded from the cervix with a vaginal approach [10,12]. One might argue if a more marked EMG signal would have been recorded from the stromal layer at a higher level of amplification, but under such conditions baseline noise is greatly enlarged and recorded EMG signals could possibly also have been originated from the surrounding CML.

The fact that the smooth muscular cells in cervical stoma are arranged in loose bundles with different orientations may also have contributed to the fact that we did not pick up a decent EMG signal. Additionally, it might be that the CSL smooth muscle cells play a role in the inflammatory cascade that leads to cervical softening [45-49].

In conclusion, during late gestation the pattern of EMG activity of the CML exhibits a pattern of contractures, highly similar to that of the MYO. During induced parturition, the EMG activity of the CML increases simultaneously with the MYO and this increase precedes the onset of cervical dilatation by some 12h. Further studies are needed to elucidate the physiological functions of this phenomenon.

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7

Cervical diameter in relation to uterine and cervical EMG activity in early postpartum dairy cows with retained placentas after PGF2α induced calvings

3

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Abstract.

The cervix must regain its normal diameter after parturition. Until now, little has been known about the pattern of cervical closure and the possible influences of myometrial and cervical contractions in this process. We continuously measured the cervical diameter with ultrasound cervimetry during the first 48 h after calving in six cows with retained fetal membranes, while uterine (n=6) and cervical outer muscular layer (n=4) electromyographic (EMG) activity was measured with bipolar EMG electrodes.

We found that the cervical diameter, which was 6.2 (± 0.7) cm at 1.4 h after calving, initially increased to 9.0 (± 1.0) cm during the first 14.8 (± 2.8) h post partum. After this time, the diameter decreased gradually to 5.3 (± 1.0) cm at 48 h after calving. The overall EMG activity after parturition decreased by 59 (± 6) % and 35 (± 17) % for the uterus and cervix, respectively. The decrease in EMG activity was due to a 50 (± 7) % decrease in EMG amplitudes of the myometrium; the EMG amplitudes of the cervix decreased by only 8 (± 21) % (P> 0.05). At the same time in the cervix, burst frequency decreased by 69 (± 17) %, while the decrease in burst frequency of the myometrium was only 11 (± 5) % (P> 0.05)

Uterine myometrial and cervical EMG activity after parturition showed burst patterns. These contractions of the uterus and cervix were accompanied by and correlated with transient dilatations of the caudal cervix. This could have functional relevance in the evacuation of the uterus.

Introduction

During pregnancy, the uterine cervix is firm and closed, due to a high content of connective tissue, which is dispersed with and surrounded by bundles of smooth muscle cells [1]. Shortly before and during parturition, the connective tissue of the cervix is remodelled [2-4] and loses its strength [5], which results in a softened cervix that is able to dilate and accommodate the fetus when the uterus contracts [6]. The cervix is an important barrier against the invasion of bacteria in the uterine cavity [7,8]. Therefore, it is obvious that closure of the cervical canal and regaining of its firm structure after parturition is important for a successful new pregnancy. When this process is incomplete or delayed, as is the case in cows with retention of the placenta, it predisposes to the development of endometritis [7,8]. Insufficient closure of the cervix thus leads to infertility or even sterility in cows [9] and mares [10].

It is evident that contractions of the pregnant uterus are involved in the dilatation of the cervix during parturition [6,11] and that equally strong and even more frequent uterine contractions are present immediately after delivery of the calf [12-21]. After uncomplicated calvings, the frequency of these contractions decreases to almost zero within two days [13,20]. In cows with retained membranes however, both the frequency and the amplitude of uterine contractions have been reported to remain high for at least more than two days [13,20]. One could question whether these post partum uterine contractions in any way interfere with closing of the cervix.

The cervix shows considerable amounts of electromyographic (EMG) activity immediately after parturition (which decreases to low levels between 48 to 72 h), as has been shown in sheep [22]. It could be expected that these post partum contractions of the cervix itself influence cervical diameter, which could either be a decrease or to an increase of the cervical diameter, as both phenomena have been described during parturition [23,24].

Given the importance of cervical closure for a good reproductive performance, it is remarkable that it has presently not been described in more detail, and that possible influences of uterine and cervical contractility

have not been explored more extensively. A better understanding of the factors that influence cervical closure would lead to a more evidence based diagnosis and intervention in cases in which this closure is impaired. In fact, there is only one report describing the process of cervical closing in cows during the first 10 days postpartum [25]. In that study, a forceps was repeatedly inserted in the genital tract. It was observed that a decrease in cervical diameter from 25 cm to 5.2 cm takes place within the first 2 days. However, no information on uterine or cervical contractility was provided in that study.

The study has the following three aims: 1) to describe the cervical diameter during the first 48 h post partum in cows with retained placentas; 2) to compare cervical with uterine EMG activity during that time period; and 3) to analyze the relationship between the EMG activity and the cervical diameter. We hypothesize that the cervical diameter, with an initial diameter of about 25 cm, decreases steadily during the first two days after calving, and that uterine and cervical EMG activity causes temporal changes in cervical diameter.

For this purpose, cervical diameter was measured continuously with ultrasound while EMG activity of the myometrium and cervix were recorded during the first 48 h after calving. This paper presents the results of a study that was performed on cows that previously had been used in an experiment in which the cervical diameter and EMG activity of the myometrium and cervix were recorded during a parturition which was induced with a synthetic PGF2α analogue [24]. As a consequence of the use of PGF2α all the cows had retained placentas, due to insufficient shedding of the cotyledons from the caruncles [20], after otherwise normal calvings.

Materials and methods

Animals and Surgery

Six pluriparous Holstein Friesian (HF) cows, bred by HF bulls, were purchased from farmers at 6-7 months of pregnancy. They were selected on the following traits: a body condition score of 3-3.5 [26], only spontaneous uncomplicated parturitions in their history and a carrying singleton pregnancy with an anterior presentation. Six cows were deemed to be sufficient for this study, based on previous studies using similar physiological parameters and experimental protocol [14,15,24,27]. The use of these animals in this experiment, which was a follow-up of a previously described study [24] had been approved by the Ethical Committee of the Veterinary Faculty of Utrecht University (The Netherlands).The cows were housed in individual stands in the clinical facilities of the veterinary faculty from their arrival until the start of the experiments. They underwent surgery 10 to 14 days before parturition was induced with a synthetic PGF2α analogue (Prosolvin ®, 2 ml im, Intervet, Boxmeer, The Netherlands,) on day 274 of pregnancy. The surgical procedure, which we describe briefly below, has been extensively outlined elsewhere [24,27]. During abdominal surgery, two bipolar AgAgCl electrodes, made in our own laboratory with 5 mm distance in between [28], were sutured on the surface of the uterine myometrium near the top of the uterine horn. A bipolar fishhook-like stainless steel electrode, prepared from two stainless steel fishhooks, (Partridge of Redditch, UK) imbedded in epoxy with 5 mm distance in between, was attached on the middle dorsal surface of the cervical outer muscular layer. The wires, connected to the electrodes, were tunneled subcutaneously and exteriorized at the dorsal sublumbar area. The cows received flunixin meglumine (1 mg/kg iv, Bedozane ®, Eurovet, Bladel, The Netherlands) for 2 days and ampicillin (10 mg/kg im, Praxavet Ampi®, Boehringer-Ingelheim, Alkmaar, The Netherlands) for 5 days. They had free access to food and water and were placed in an individual pen (3.1 m^2) after 2 days of recovery. They were tethered with a rope preventing them to reach the cables between them and the equipment.

Experimental protocol

The cows were under continuous video observation and none of them showed signs of dystocia. All cows delivered a healthy Holstein Friesian calf in anterior presentation, at an average interval of 38.8 (± 3.4) h after PG administration. Almost immediately after vaginal expulsion of the calf, two ultrasound transducers as manufactured and described by Eijskoot et al [29], were sutured to the internal aspect of the caudal cervical rim. They were placed opposite to each other; at 9 and 3 o'clock positions using a vaginal approach after caudal epidural anaesthesia had been applied to avoid abdominal straining during the procedure. One of the transducers functioned as transmitter and the other as receiver. The signals from the cervimeter were digitized with a multichannel analog/digital converter (National Instruments, Austin, USA), sampled with 40 Hz and stored with Labview 5.0 software (National Instruments, Austin, USA). After having sutured the transducers in place (at 1.2 to 1.9 h after birth of the calf), the cervical diameter and the EMG activity of the uterine myometrium and cervical muscular layer were measured continuously until 48 h after expulsion.

Because the cows were expected to retain their placenta, a capsule with tetracycline (2 gr, Virbac, Barneveld, Netherlands) was manually placed in the formerly pregnant uterine horn, immediately after the ultrasound transducers had been sutured onto their position. The calves removed from their mothers immediately after birth and the cows were milked twice per day. Each milking took only a few minutes and the data obtained during these short periods were included in the analysis.

The signals from the EMG electrodes were amplified with two amplifiers (model 11-4123-01; Gould Inc., Cleveland, OH or model UME-47; Schwarzer, München, Germany) and analog bandpass filtered from 0.05 up to more than 10 Hz. All signals were subsequently digitized with a multichannel analog/digital converter, sampled with 40 Hz and stored with Labview 5.0 software. The data from the EMG activity recordings were digitally high-pass filtered (0.125 Hz) in Labiew to correct for slow fluctuations in tissue voltage.

Data analysis and Statistics

Cervical diameter

The mean cervical diameter was calculated for consecutive 10 sec periods. Plotting cervical diameter against time revealed two different stages; a first stage during which the cervical diameter did not decrease but in fact even increased to a maximum value, and a second stage during which the cervical diameter progressively decreased. This pattern was analysed with bi-linear regression analysis in SPSS (version 12.0.1, Chicago, USA, 2003) as previously described for the analysis of cervical dilatation in parturient cows [15]. The bi-linear regression analysis was used to calculate the time after delivery at which the postpartal cervix diameter reached its maximum and the magnitude of that maximum. As Fig 3.1 exemplifies for one cow, the calculated values describe the raw data accurately. The parameters were first obtained for each cow separately, after which overall means (±SEM, n=6) were calculated.

Uterine and cervical EMG activity

The EMG activity pattern of the myometrium and cervix post partum differed from ante partum and was characterized by well-defined bursts, alternated with periods of inactivity. As a result, we were able to analyze the EMG activity of the myometrium and cervix quantitatively using three different parameters.

Firstly, the total EMG activity was quantified by calculating the root mean square values (RMS) over consecutive 10 sec periods. Due to the presence of noise, the RMS values appeared to have a basal level; this basal level was determined for each cow separately and subtracted from the values during the entire recordings. Secondly, the maximal positive EMG amplitude was determined for each consecutive 15 min period. Thirdly, the number of bursts of EMG activity was counted for each consecutive 15 min period.

The changes in the RMS values of EMG activity, the numbers of bursts and the maximal EMG amplitudes for the first 48 h after calving were further quantified with linear regression analysis in SPSS. The relative change in EMG activity was calculated by dividing the regression coefficients, which

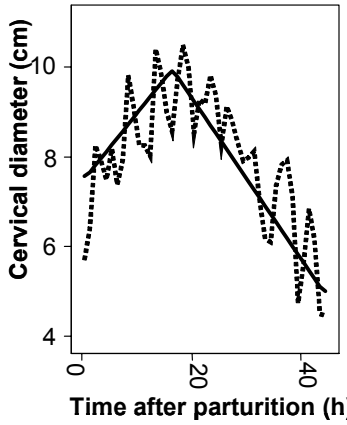

Figure 3.1. An example of hourly measured values of cervical diameter in an individual cow (- - - -), and calculated bi-linear regression lines (____). The bi-linear regression analysis was used to calculate the maximum cervical diameter and the time at which the maximum was reached.

reflected the changes in EMG activity in time, by the initial values of the EMG activity. The parameters were first obtained for each cow separately, after which overall means (±SEM, n=6) were calculated. A one-sample t-test of the means of the individual cows was used to assess if there were significant changes in EMG activity. Values were regarded as statistically significant when P was lower than 0.05.

Additionally, the relationship between the occurrence of individual EMG bursts and transient changes in cervical diameter was analysed. For this purpose, correlations were calculated between the RMS values of the EMG activity of the uterus and cervix and the cervical diameter. These correlations could be analyzed on two time scales. Firstly, the long-term relationship between the course of EMG activity and cervical diameter over the 48 h period. Secondly, the short term relationship between individual bursts of EMG activity and simultaneous occurring changes in cervical diameter (temporal effects). Because we were primarily interested in the short term correlation, it was necessary to eliminate the influence of the long term correlations. This was done by correcting the correlation coefficients for time effect and cow effect, with the partial correlations method in SPSS.

Results

Each cow delivered a healthy calf in anterior position and retained the fetal membranes for more than 3 days. It took between 1.2 and 1.9 h after birth of the calf before the ultrasound transducers were sutured to the caudal cervical rim, and the continuous measurements started. In two cows, the cervical muscular layer electrodes did not detect an EMG signal after calving, because they had become disconnected, probably because they were damaged during fetal expulsion. Examples of the original EMG activity of the myometrium and cervical muscular layer, as well as changes in the cervical diameter are given in Fig 3.2 (all data obtained from the same cow at different time periods after calving).

The cervical diameter

The cervical diameter was 6.2 (± 0.7) cm at the start of the measurements, which was on average at 1.4 h after expulsion of the calf (Fig 3.3A). Quite unexpectedly, there were two stages to be recognized. With bi-linear regression analysis, we calculated that the first stage took 14.8 (± 2.8) h, during which the diameter of the cervix increased ($P< 0.05$) to 9.0 (± 1.0) cm; the second stage took 33.2 (± 2.8) h, during which the cervical diameter decreased ($P<0.05$) to 5.3 (± 1.0) cm at 48 h after expulsion of the calf, which was the arbitrarily chosen moment that we ceased the measurements. At that time the cervical diameter ranged between cows from 2.0 to 9.3 cm.

The EMG activity of the cervical muscular layer and myometrium

Immediately after parturition both the myometrium (n=6) and cervical muscular layer (n=4) showed EMG activity, which consisted of individual bursts with periods of rest in between. Bursts derived from the myometrium were uniform in duration and amplitude. Bursts from the cervical muscular layer however, were largely variable in duration and amplitude (Fig 3.2A). In time, the amplitudes of the EMG activity of the myometrium decreased to very low levels in all cows, but the amplitudes of the EMG activity of the cervical muscular layer did not clearly decrease. The frequency of bursts of the myometrium remained at about the same level during the 48 h period of

the measurements (Fig 3.2A, B and C) but in the cervical muscular layer, the frequency of bursts decreased over time.

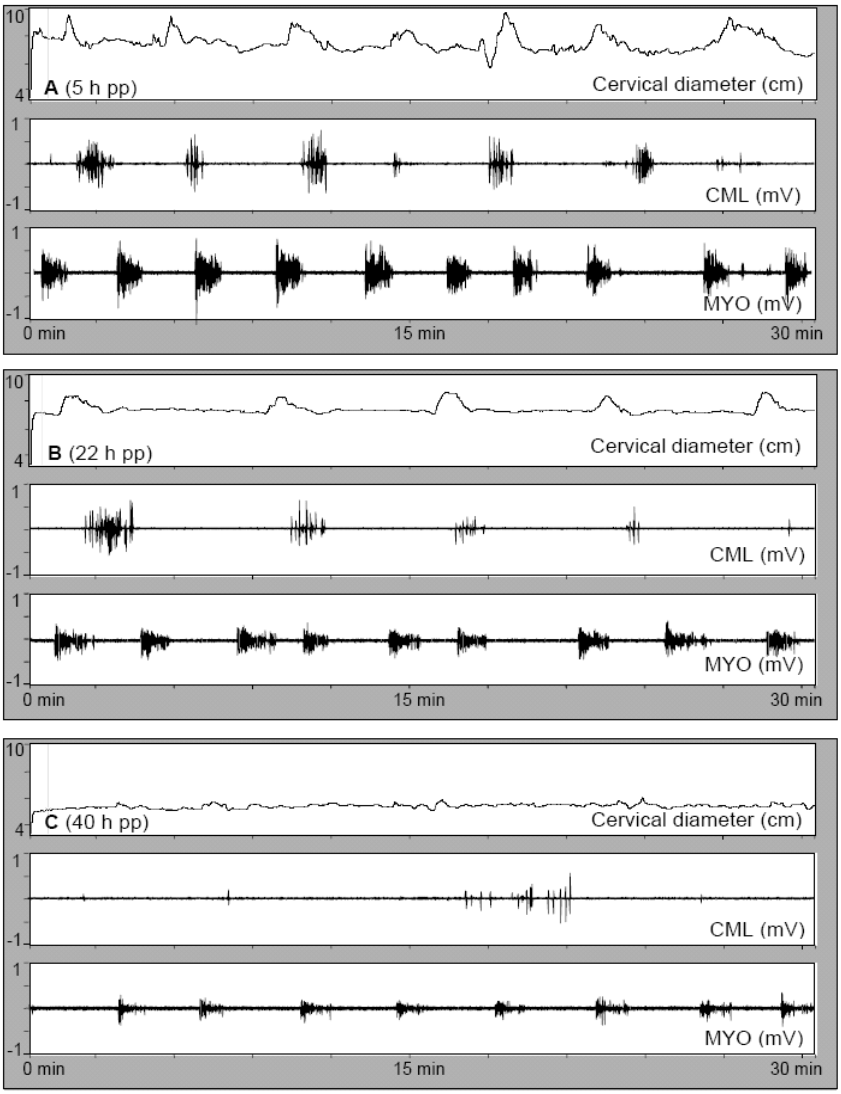

Figure 3.2. Representative examples of 30-min recordings of the cervical diameter (cm) and EMG activity (mV) of the cervical muscular layer (CML) and myometrium (MYO) at A) 5 h; B) 22 h and C) 40 h post partum. The examples were derived from the same cow.

The myometrium showed a total decrease of 59 (± 6) % (P< 0.01) of the RMS values over the 48 h postpartum period (Fig 3.3B). The cervical muscular layer however showed a total decrease of 35 (± 17) % (P> 0.05)) of the RMS values (Fig 3.3C).

Maximal EMG amplitudes of the myometrium decreased with 50 (± 7) % (P< 0.01) over the 48 h postpartum period (Fig 3.4C). Maximal EMG amplitudes of the cervical muscular layer varied largely but did not change in time (8 (± 21) %, (P> 0.05)), despite what may be suggested by Fig 3.4D.

The myometrium showed a burst frequency of 19.8 (± 2.2) per h during the first h of our measurement (Fig 3.4A), after which it decreased with 11 (± 5) % (P> 0.05) in total. The cervical muscular layer showed an initial burst frequency of 15.4 (± 1.3) per h (Fig 3.4B), after which it decreased with 69 (± 17) % (P< 0.01). The mean burst frequency in the myometrium was higher than that in the cervical muscular layer. This difference became significant (P<0.05, repeated measures) from 29 h after parturition onwards. Despite this difference in mean burst frequencies, the bursts of both tissues showed synchrony. This mirrored the positive correlation 0.178 (P< 0.001) between the RMS values of the two sites.

The relationship between the EMG activity and cervical diameter

In all cows, the cervical diameter increased transiently during EMG bursts throughout the first day post partum, as illustrated for one cow in Fig 3.2. These transient dilatations of the cervix occurred more often synchronously with a burst of the cervical muscular layer than with a burst of the myometrium. During the second day post partum not only did the baseline cervical diameter decrease but also the magnitude of the transient increases in cervical diameter. These temporal dilatations disappeared completely after 36.2 (± 2.7) h.

The diameter of the cervix was positively correlated with the level of EMG activity (RMS values) in all cows. When the correlations (r) were corrected for time effect, the correlation between the actual cervical diameter and the cervical muscular layer EMG activity was 0.255 (P<0.001, two-tailed analysis) which was larger (P<0.05) than the correlation between the actual

cervical diameter and the myometrial EMG activity which was 0.168 (P<0.001, two-tailed analysis).

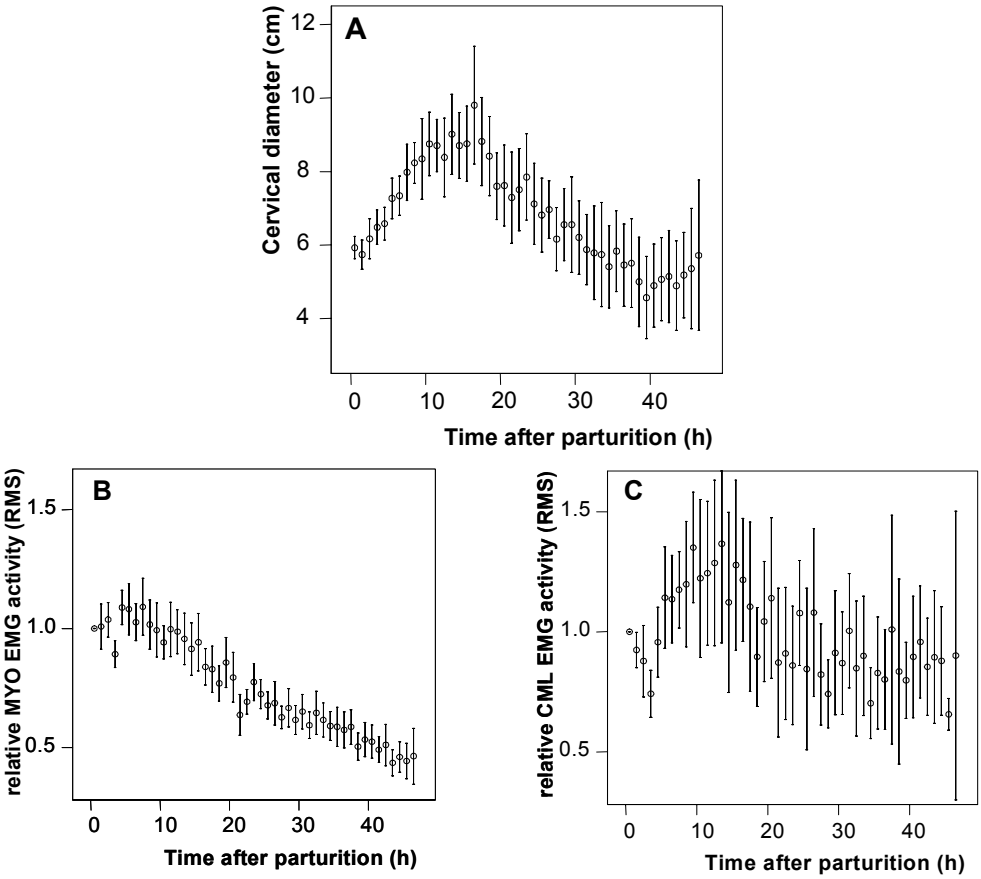

Figure 3.3. Mean values per h (± SEM) of cervical diameter (cm) and EMG activity during the first 48 h after calving. A) The cervical diameter (n=6); B) The EMG activity of the myometrium (MYO; n=6) expressed as RMS values; C) The EMG activity of the cervical muscular layer (CML; n=4) expressed as RMS values. EMG activity is expressed relative to the values during the first h of the measurements. Expulsion of the calf occurred at t=0.

Discussion

Diameter of the cervix

Much to our surprise, the diameter of the caudal cervix had already markedly decreased to 6.2 cm at the beginning of our measurements, i.e. 1.4 h after calving. In three different studies, we have been able to measure the cervical diameter until the time that progression of fetal parts into the cervical canal disturbed the measurements. At that time, 15 out of 16 cows showed a cervical diameter of between 14 and 20 cm. [15,24,27]. Also others described a cervical diameter of 20 cm at 1.5 h after spontaneous parturition [25]. Collagen breakdown is the main contributor to the increased softness of the cervix around parturition [2-4], but it would not be expected that in such a short time new collagen fibrils would have lead to stiffening of the once-flaccid tissue and contributed to this speedy recovery of the diameter. Besides collagen, the cervical stromal layer contains much smooth muscular tissue [24] which contracts under influence of oxytocin in vitro. Therefore an active muscular component originating in the cervix could be responsible for this considerable decrease. Stretching of the cervical and vaginal tissues by the passage of the calf or the manipulations during suturing of the ultrasound transducers may have caused such muscular activity. However, we cannot verify this because no EMG data are available from the first 1.4 h after parturition. Another possible explanation could be that the initial decreased cervical diameter after calving is caused by a passive collapse of the flaccid walls of the cervical canal, which is not occupied by the fetus anymore. Shortly after parturition, the caudal birth canal is distended. This leads commonly to aspiration of air, when the cow is in a standing position. Air becomes trapped in the vagina and uterus and might prevent cervical collapse. Normally the cow expels this air by uterine contractions and abdominal straining and this happens quite rapidly under influence of the high abdominal pressure when the cow is in a recumbent position. This expulsion of air may explain the rapid return of the cervical diameter to one of about 6 cm, such as we have measured. On the other hand, repeatedly measuring the cervical diameter of early post partum cows with calipers [25], which have to be introduced in the vagina while the cow is standing may cause aspiration of air again and again [30], which

could explain why a diameter of 20 cm was measured by Wehrend at al [25].

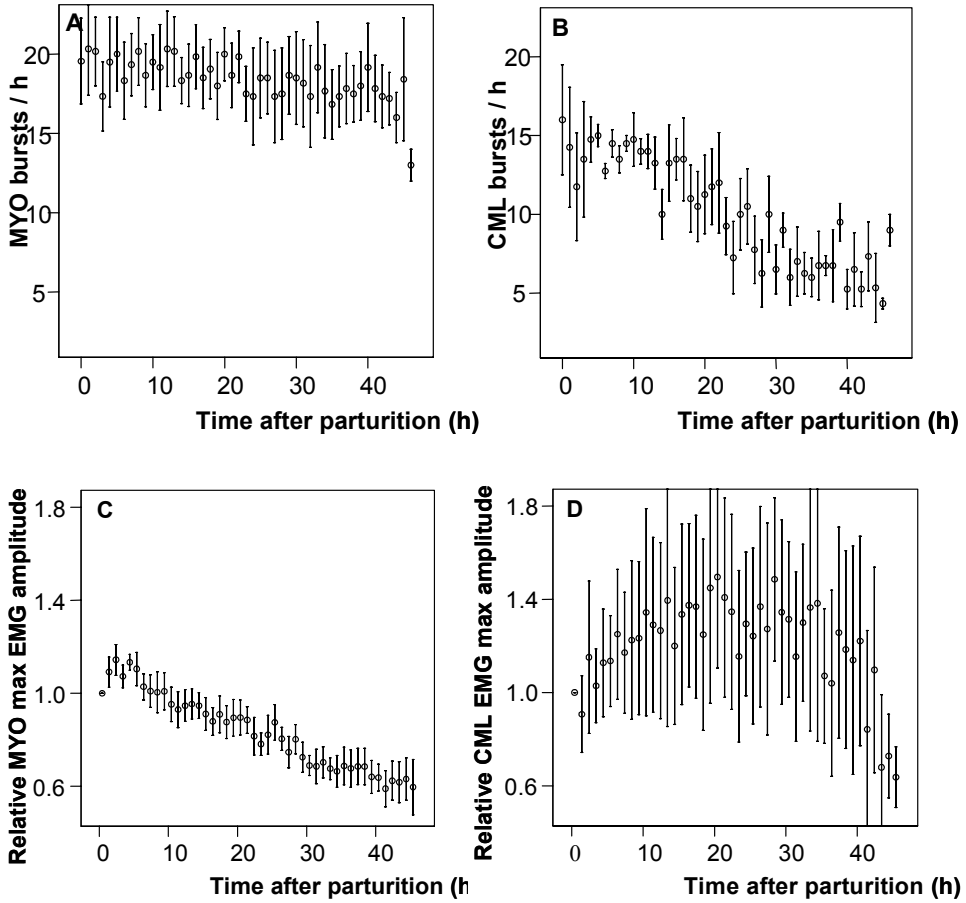

Figure 3.4. Mean values (± SEM) of; A) burst frequency per h of the myometrium; B) burst frequency per h of the cervical muscular layer; C) maximal EMG amplitude of the myometrium and D) maximal EMG amplitude of the cervical muscular layer. The data are obtained from continuous measurements of EMG activity of the myometrium (MYO; n=6) and the cervical muscular layer (CML; n=4) during the first 48 h after calving. Values for maximal EMG amplitudes are expressed relative to the value for the first h of the measurements. Expulsion of the calf occurred at t=0.

Even more unexpectedly the cervical diameter did not decrease further to a diameter smaller than 6 cm, but instead it increased up to 9 cm, during the first 15 h post partum. Directly after a normal parturition, the uterus is empty, but still large in size. Thus, the placenta, with the placentomes still intact and attached to the uterine wall, lay far away from the cervix. Because the uterine cavity decreases rapidly in size after expulsion of the calf, the placenta comes more close to the cervix, which is supported by our observation, that parts of the placenta became visible outside the vulva in two cows. The presence of the placenta could force open the still soft and pliable cervix. One may ask if an increase in cervical diameter also takes place in cows without placental retention; one would expect that the cervical diameter is increased at the time that the placenta is expelled, but such measurements have not yet been reported.

The cervical diameter started to decrease again at 15 h after parturition. During the period thereafter, the mean cervical diameter and the rate of its decrease are in accordance with the results of Wehrend et al [25], who measured similar diameters in cows without retained placentas. During this stage, the presence of a retained placenta in our study seemed not to have influenced the cervical diameter any longer.

The EMG activity of the cervical muscular layer and myometrium

Although uterine and cervical EMG activity were correlated and both showed a decrease in RMS values during the first 48 h post partum, cervical EMG activity also showed clearly several differences compared to uterine EMG activity. Firstly, the pattern of bursts in the cervical muscular layer was less well organized than in the uterus. The pattern of bursts of EMG activity of the myometrium was in accordance with previous reports for the postpartum period after PGF2α induced calving [14]. These bursts are usually associated with contractions, as reflected by changes in intra uterine pressure [14,31]. The observation that the pattern of EMG activity of the cervical muscular layer appeared to be less well organized, corresponds with our findings in the same cows before fetal expulsion [24] as well as with findings in postpartum sheep [22]. Secondly, the decrease of the RMS values and maximal EMG amplitudes in the cervical muscular layer was less substantial than in the uterus and showed much variation

between cows. RMS values and maximal amplitudes of the EMG activity both reflect the rate of synchronization of electrical events between smooth muscle. The formation of gap junctions is involved in this phenomenon, and is regulated by estrogens and prostaglandins [32,33]. It has been shown, that the regulation of the gap junction formation is different for the two layers of the myometrium [34]. A similar difference could also exist between the myometrium and cervical muscular layer, which would explain the difference between changes of RMS values and EMG amplitudes of myometrium and cervical muscular layer.

Thirdly, the frequency of the bursts of EMG activity in the cervical muscular layer was lower than in the myometrium. This agrees with a study in parturient cows that showed a decline in the frequency of contractions from the uterine horn towards the uterine body [31]. Although the parturient uterus may be considered as a functional syncitium [35,36], not all contractions that originate at the tip of the uterine horn will reach the uterine body and the cervix. This results in lower contraction frequencies at more caudal sites of the genital tract. A possible functional significance of this phenomenon is to create a pressure gradient within the uterus that enables the uterus to evacuate itself, even after expulsion of the calf. In the cow, the propagation of contractions from the tubal- to the cervical end of the uterus takes some 25 sec, depending on the stage of calving [31]. This explains why we found that, although bursts of EMG activity of the myometrium and cervical muscular layer were positively correlated, they had a low correlation coefficient compared to the one (0.6) that has been reported by Bajcsy et al [14]. The latter authors determined the post partum EMG activities at two sites along the longitudinal axis of the myometrium, with a distance of approximately 30 cm between the electrodes.

Fourthly, The frequency of bursts decreased in the cervical muscular layer, but remained high in the uterus which is in accordance with earlier reports from cows with retained placentas [13,20]. It has been shown before that the cervix displays its own contractility pattern during parturition and that its response to prostaglandin-E and oxytocin differs from that of the uterus [22,24,37]. Our study shows that such differences in contractility also exist

after parturition and further emphasizes that the cervix has to be regarded as an organ with own function acting partly independent of the uterus.

The relationship between EMG activity and cervical diameter

One of the aims of the present study was to investigate to which extent the cervical muscular layer or myometrial activity influences the diameter of the cervix during the early postpartum period. Immediately and shortly after fetal expulsion, EMG bursts of the myometrium and cervical muscular layer were accompanied by transient increases of the cervical diameter. This reflects a cervical tissue that is still soft and pliable. It also suggests that during this early postpartum period, contractions of the uterus and cervix facilitate the expulsion of the (retained) fetal membranes, by a combination of reducing the uterine cavity and instantly increasing the cervical diameter. The gradually decreasing cervical diameter after 15 h post partum and the decreased magnitude of the temporal cervical dilatations during contractions point to a more rigid state of cervical tissue, but could also follow from a combination of a weaker uterine contractility and a less frequent cervical muscular activity. Our observation that changes in the cervical diameter had a stronger positive correlation with the EMG activity of the cervical musculature than with that of the myometrium, suggests that during the early post partum period cervical contractions play a more important role in keeping the cervix open than myometrium contractions, while the latter primarily cause a pressure build up and facilitate expulsion of the uterine content. But regarding the weak nature of the correlations, it is likely that other factors may play a more decisive role in the regulation of the cervical diameter post partum.

With this study, we have demonstrated that in cows with retained placentas cervical closing during the first 2 days post partum takes place in two stages and that the cervical diameter is influenced by cervical and uterine contractions. However, more study is needed to assess the causal relationship between cervical and uterine contractions and the cervical diameter.

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7

Smooth muscle cells of the bovine cervical stroma may have a secretory, rather than a contractile function during parturition

4

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Reproduction in Domestic Animals

Abstract

The bovine cervix contains a large amount of smooth muscle cells distributed over an outer muscular layer and within a stromal layer. The stromal layer exhibits no EMG activity at parturition. This leads to the question whether the stromal smooth muscle cells of the bovine cervix are prepared to contract with parturition, or whether they have another function. To this end, cervical biopsies were repeatedly taken from 10 pregnant cows at day 185 and day 275 of gestation, at spontaneous, uncomplicated calving and at 30 days after calving. The smooth muscle bundles of the stroma were immuno histochemically analysed (n=5) with regard to their integrity and cellular density, and the degree of staining for connexin-43, smooth muscle actin α (SMA), desmin and vimentin. Additionally, the mRNA expression for connexin-43, SMA, desmin and vimentin was determined with RT-PCR (n=5). The smooth muscle tissue was arranged in bundles, also at parturition. However, the cellular density of these bundles and the SMA mRNA expression were decreased at parturition. Additionally, the SMA staining and connexin-43 expression and staining remained constant during pregnancy and at parturition. This might indicate that stromal smooth muscle cells are not prepared to contract with parturition, in contrast to the myometrial smooth muscle cells. The smooth muscle cells, stained for SMA, also expressed vimentin, and the proportion of co-expression was increased at day 275 of pregnancy. This suggests that the stromal smooth muscle cells predominantly have a secretory function in cows.

Introduction

The bovine cervix has a high muscular content [1]. The distribution of smooth muscle cells over an outer muscular layer and within the deeper part of the stromal layer has been demonstrated using immuno-histochemical staining for smooth muscle actin α (SMA) [2,3]. The smooth muscle cells within the stromal layer are grouped in small bundles with a random orientation, embedded in a connective tissue meshwork [3] and it has been observed that these smooth muscle bundles contract in response to oxytocin *in vitro* [4].

Cervical muscular contractions have been reported in human, rat, sheep and cow cervices, both *in vitro* [5-7] and *in vivo* [4,8,9]. However, the physiological relevance of cervical smooth muscle contractility has been questioned for the rabbit, rat and human because of the low density of smooth muscle cells and on the basis of contraction studies [10-12]. Nevertheless, cervical contractions in humans were correlated with a delay in cervical dilatation during parturition [13]. This suggests that cervical muscular activity actually inhibits cervical distension during pregnancy and parturition [8] and seems to cause retention of the foetus even once the extracellular matrix has lost its strength [14]. The description of cervical EMG activity during parturition in humans contrasts with the absence of EMG activity of the cervical stroma in cows [4]. In cows, this absence of EMG activity also apparently contrasts with the vast increase in EMG activity in the myometrium during parturition. The increase in EMG activity in the myometrium depends on the presence of gap junctions as is shown in parturient sheep [15] and parallels with a large increase in connexin-43 mRNA and protein expression, as is extensively shown for rats [16]. Several factors within the cervix might explain this difference; the number of smooth muscle cells could be decreased with parturition or the bundle structure of the smooth muscle could be lost. It is also possible that the expression of SMA and connexin-43 in smooth muscle cells fail to increase at that time, which would prevent these cells from contracting in a coordinated fashion, such as occurs in the myometrium [15,17].

It has been shown that besides smooth muscle cells which display a contractile function, other smooth muscle cells predominantly have a secretory function. These two types of smooth muscle cells have been identified in vascular smooth muscle [18,19] based on their phenotypic appearance. The contractile phenotype was characterised by an abundant actin and myosin content, while the secretory phenotype showed a prominent endoplasmic reticulum and Golgi complex. Recently, both phenotypes have also been identified in the rat vagina, the contractile phenotype being more prominent in virgin and postpartum rats while the secretory phenotype dominated during pregnancy [20]. The smooth muscle cells of the visceral organs express SMA and desmin but predominantly lack vimentin [21]. In contrast, vascular smooth muscle cells with a secretory phenotype also express vimentin [18], which has been associated with secretion of matrix metallo proteinases (MMPs) [19]. The cervical extra cellular matrix (ECM) is largely remodelled during parturition, and in humans, cervical smooth muscle cells secrete MMPs which are involved in this process [22]. Therefore, it is of functional interest to determine whether the phenotype of stromal smooth muscle cell phenotype is predominantly a contractile or a secretory one, and if the ratio between the two is dependent on the stage of pregnancy. Therefore, we studied whether the smooth muscle bundles of the cervical stroma co-express vimentin and SMA and whether the expression of vimentin, desmin or SMA changes with the stage of pregnancy. We also studied the expression of connexin-43 because this indicates the number of gap-junctions present, which is an indication of the contractile activity of smooth muscle cells.

Serial biopsies of the bovine caudal cervix were carried out at days 185 and 275 of pregnancy, at parturition and at 30 days postpartum. They were subsequently analysed by means of immunofluorescent double-staining and RT-PCR.

Materials and Methods

Animals

Cervical biopsies were collected from 10 pluriparous Holstein Friesian cows transvaginally using a skin biopsy punch of 6 mm diameter (Kai industries co. Ltd. Oyana, Japan) as described before [23]. The experimental procedure was approved by the Committee For Use of Animals in Research at Utrecht University. The cows belonged to a commercially kept, high yielding dairy herd and were housed at the experimental farm of the Faculty of Veterinary Medicine at Utrecht University. They were fed according to their individual needs as defined by their level of milk production and stage of gestation.

Biopsy collection

The biopsies were obtained from the inside of the cervical canal, approximately 2 cm cranial to the caudal cervical opening and included both the superficial and the deep stromal layer (Fig. 4.1). The first biopsy was collected at 185 (± 3) d and the second at 275 (± 1) d of gestation. The third biopsy was collected at calving, on average within 2 (± 2) h after spontaneous uncomplicated vaginal delivery of the calf, before shedding of the foetal placenta. The fourth biopsy was collected at 30 (± 3) d after calving. The biopsies were cleaned of blood and mucus and cleaved lengthwise. One part was snap frozen in liquid nitrogen, and stored at -80 °C until further analysis. The other part was fixed in 4.5 % formaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. The size of the cleaved biopsy was too small to allow all analytical procedures on each sample, therefore the RT-PCR and immuno histochemical stainings were performed with two different sets of 5 cows.

RT-PCR for SMA, connexin-43, vimentin and desmin

The frozen tissue samples were pulverised with a mortar and pestle. Subsequently, RNA was isolated using RNeasy Fibrous Tissue Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. In short, the pulverised sample was transferred to 300 µl lysis buffer, drawn through a 20 gauge needle and subjected to proteinase K

treatment (10 mAU/ml) for 10 min at 55 ºC, followed by centrifugation for 3 min at 1000 g. The supernatant was run through a Qiagen mini column, after which the column was washed twice. RNA was eluated from the spin column with 50 µl RNAse free water. RNA was quantified by spectrophotometry (260 nm) and evaluated for degradation on a 1 % agarose gel. The RNA samples were stored at -80 °C until further use.

Protein	Forward primer '5- -3 ' Reverse primer '5- -3'
GAPDH NM_001034034	F: AAG TTC AAC GGC ACA GTC AAG G R: CAT ACT CAG CAC CAG CAT CAC C
Connexin-43 NM_174068	F: CTT ATT TCA ATG GCT GCT CCT C R: TGC TCA CTT GCT TGT TTG TTG
Smooth muscle actin α BT 021508	F: AGG GAG TGA TGG TGG GAA TGG R: GTG ATG ATG CCGTGC TCT ATC G
Desmin NM_001081575	F: GAC CCA GGC AGC CAA CAA G R: GTC GAT CTC GCA GGT GTA GG
Vimentin NM_173969	F: GAC CTG GAG CGT AAA GTG G R: GAC ATG CTG TTC TTG AAT CTG G

Table 4.1 Forward and reverse primers and accession numbers of the genes which are used for RT_PCR analysis of bovine cervical tissue.

cDNA was synthesized with the iScript™ cDNA Synthesis kit (Biorad, Luxembourg) according to the manufacturer's instructions. Briefly, 5 µg RNA was added to 4 µl iScript Reaction Mix (Biorad, Luxembourg), 1 µl iScript Reverse transcriptase (Biorad, Luxembourg) and RNase free water to a total volume of 20 µl. The complete reaction mix was incubated in a thermalcycler (Mycycler, Biorad, Luxembourg) for 5 min at 25 ºC and 30 min at 42 ºC, after which the enzymes were inactivated for 5 min at 85 ºC. Samples were diluted to 10 ng/µl working solutions and stored at 4 ºC.

Real-time PCR was performed with a MyiQ system; iCycler and detection unit (Biorad, Luxembourg) using SYBR-Green Supermix (Biorad,

Luxembourg); cDNA input was 50 ng and the concentration of the forward and reverse primers was 330 nM. For the real-time PCR, the sequences of the forward and reverse primers for GAPDH, connexin-43, SMA, desmin and vimentin are listed in Table 4.1. Product specificity of the primer set was verified by melting curve analysis after the PCR had finished. For each sample, duplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model for the relative expression ratio in real-time PCR, the $2^{-\Delta\Delta Ct}$ –method [24,25]. An earlier study of the cervix of pregnant and parturient cows showed that the expression of GAPDH was highly correlated (r = 0.9) with the expression of actin-β [2], therefore GAPDH was regarded as a household gene and used as reference.

Immuno histochemistry and histometry for SMA, connexin-43, vimentin and desmin

The following primary antibodies were used for the immuno histochemistry and fluorescent labeling: Rabbit anti-GapJunction A1 (connexin-43) (1:150, Abcam, UK); Rabbit anti-desmin (1:100, Cappel, USA); Rabbit anti-smooth muscle actin (1:300, Neomarkers, USA); Mouse anti-smooth muscle actin (1:200, DakoCytomation, Denmark); Mouse anti-vimentin: (1:100, DakoCytomation, Denmark).

The 5 µm thick length-cut sections were mounted on a coated glass slide (Superfrost plus, Erie Sc. Co., Portsmouth, USA). The sections were deparaffinised and rehydrated with xylene and a series of ethanols and rinsed twice with PBS/ Tween-20. Sections for connexin-43 staining were pre-treated with 0.1% Trypsin at 37 ºC. The sections were incubated with normal goat serum (NGS) in PBS (1:10) for 30 min to block non-specific binding of the antibodies. Subsequently, they were incubated with a mixture of the primary antibodies for 60 min and washed three times with PBS/ Tween-20. Finally, they were incubated for 60 min with a mixture of goat anti-rabbit Alexa 488 (1:100, Molecular probes, USA) and goat anti-mouse Alexa 568 (1:100, Molecular probes, USA) in PBS with 10% NGS. After washing three times in PBS and incubation with 4,6-diamidino-2-phenyl-indole (DAPI) (300 nM, Vector Labs, USA) in PBS for 20 min, they were washed again three times in PBS and mounted with Fluorosave (Cal-biochem, Germany). Control sections were incubated with the

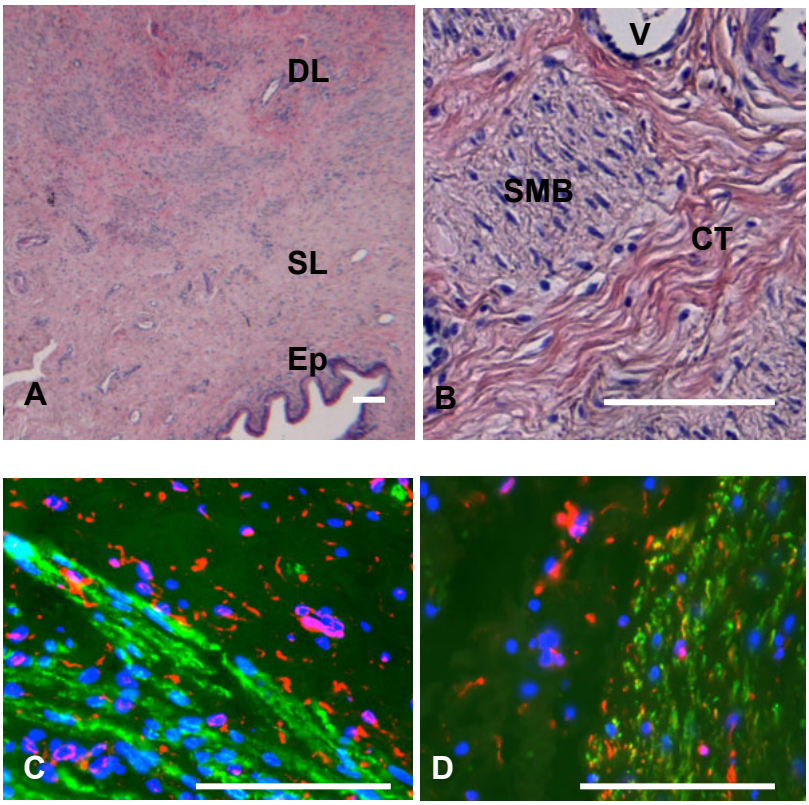

Figure 4.1 A and B: General morphology of a biopsy from the bovine cervix (30 d after parturition, HE staining). A) showing epithelium (Ep), superficial stromal layer (SL) and deep stromal layer (DL); B) detail view of the deep stromal layer showing smooth muscular bundle (SMB), connective tissue (CT) and vasculature (V). C and D: structure of the smooth muscular bundles in the deep stromal layer with double fluorescent staining for SMA (green) and vimentin (red) and nuclear staining with blue (DAPI) at 30 d after parturition (C) and at parturition (D). Bar = 100 μm.

secondary antibodies only, or with a single primary antibody and both secondary antibodies. The sections were used for semi quantitative determination of labelling efficiency using a Leica DMRE fluorescence microscope with Photometrics Coolsnap CCD digital camera, equipped with IPlab software (Scanalytics, Billerica MA) using a 40 x objective and oil emersion. For all sections of each staining, images were acquired in one session using identical camera and microscope settings. Three photo-

graphs, each covering an area of 172.5 μm by 217.8 μm, were taken of each of the superficial- and the deep stromal layer at each biopsy moment.

Image analysis of the photographs and calculations

Cellular densities of the smooth muscle bundles and the connective tissue within the deep stroma and of the superficial stromal layer were determined for the biopsies of all 10 cows. The cellular density was calculated by counting the number of nuclei stained by DAPI nuclear staining, and dividing this by the total area that was covered by the tissue in the photographs.

Images were analysed using ImageJ (NIH, USA) [26], resulting in a semi-quantitative assessment of marker presence. Mean fluorescence of each staining was calculated by dividing the total amount of background-corrected fluorescence by the surface of that area, and called optical density (OD). The smooth muscle bundles were analysed in detail. For that purpose, the bundle area of the deep stromal layer was selected within the photograph, and the OD of smooth muscle actin, vimentin and desmin were assessed for that area. Also, the amount of co-expression of vimentin with SMA was determined. To do so, the SMA positive area was selected, and the vimentin OD in this area was divided by the vimentin OD of the SMA negative area.

Statistical analysis

The differences between the successive biopsies from 5 different cows were statistically analysed with the repeated measures procedure in SPSS (version 12.0.1, SPSS Inc., USA) after log transformation. When there was a significant time-effect, paired t-tests were used as post-hoc test. In cases where a significant time-effect was not shown; paired t-tests were used with Bonferonni correction. For the figures, the values in the different samples within each cow were related to the value at 185 d of pregnancy in that cow, which was set at 100%. The results are presented as means ± SEM and were regarded to be significant if $p<0.05$.

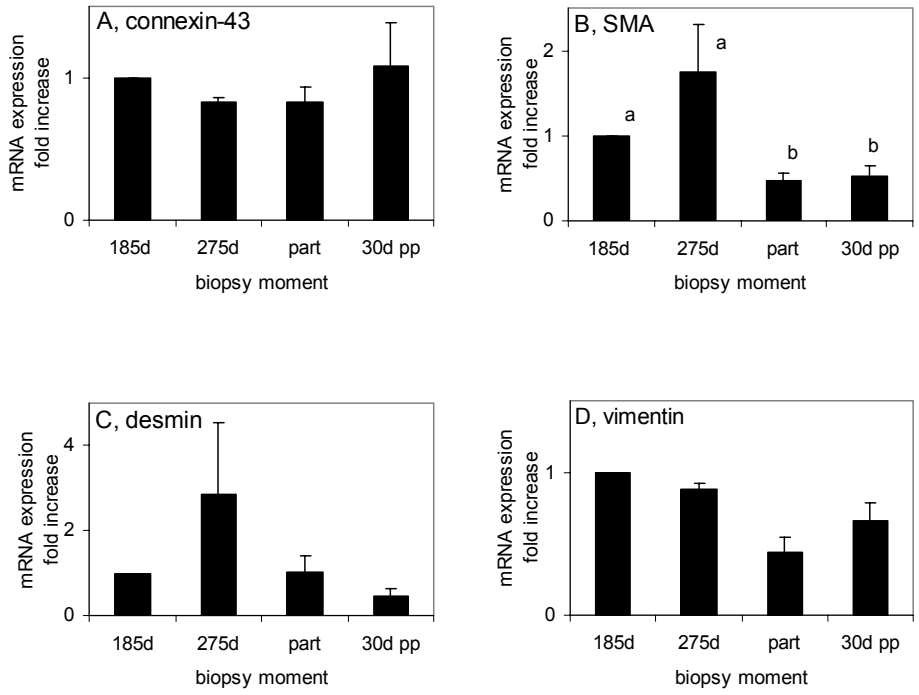

Figure 4.2 Mean relative (± SEM) mRNA expression of connexin-43 (A), SMA (B), desmin (C) and vimentin (D) in bovine cervical biopsies, relative to the expression at 185d of pregnancy. Biopsies were obtained from 5 cows at 185d of pregnancy, 275d of pregnancy, at parturition and 30 d after parturition. Values with different letters: $p<0.05$.

Results

mRNA expression

The connexin-43 mRNA expression did not change with advancing pregnancy and parturition (Fig. 4.2A). The SMA mRNA expression was higher at 185d- and 275d pregnancy compared to at parturition and 30 d after parturition ($p<0.05$, Fig. 4.2B). The desmin mRNA expression did not change with advancing pregnancy and parturition (Fig. 4.2C) and this was also observed for vimentin mRNA expression (Fig. 4.2D).

Cellular density

During the two stages of pregnancy and at 30 days after calving, the smooth muscle bundles had a higher cellular density than the connective tissue meshwork and the superficial cervical stroma ($p<0.05$, Fig. 4.3), however at parturition there was no difference in cellular density between the three components of the cervical stroma. At parturition, the relatively increased cellular density of the superficial stroma was mainly caused by an increased number of leucocytes. The cellular density of the smooth muscle bundles started to decrease between 185d and 275d pregnancy and this decrease reached significance at parturition. At 30 d after parturition, the cellular density of the smooth muscle bundles and the connective tissue between the bundles had increased to levels exceeding that during pregnancy and parturition ($p< 0.05$).

Immuno fluorescent staining of connexin-43, SMA, vimentin and desmin

Connexin-43 staining

Connexin-43 staining was abundantly present in both the superficial and the deep stromal layer. Stromal, epithelial and vascular cells (Fig. 4.4A and B) were connexin-43 positive. The staining had a granular appearance within the cytoplasm which appeared as green spots against a red background in Figure 4.4A and suggests staining of Golgi vesicles. In addition, the connexin-43 staining surrounded the SMA cytoplasmic staining with a punctate pattern which suggests that connexin-43 stained gap junctions in the cell membrane. Determination of the connexin-43 OD showed that the smooth muscle bundles expressed more connexin-43 than the superficial stroma at all stages ($p<0.05$, data not shown). The connexin-43 OD of the smooth muscle bundles did not differ between the different time-points (Fig. 4.5A).

SMA staining

SMA staining was abundant in the deep stroma (Fig. 4.4C). It was not evenly distributed within the cells but was predominantly present in the peripheral cytoplasm. The SMA positive cells formed bundles (of about 100 cells in diameter) with random orientation and separated by SMA negative connective tissue, which contained blood vessels. The structure of these

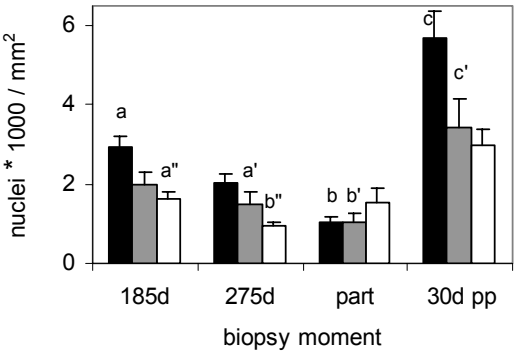

Figure 4.3 Cellular density in different regions of the cervical stroma at different time points (n=10). Smooth muscular bundle area within the deep stromal layer (▉), connective tissue area within the deep stromal layer (▒) and superficial stromal layer (□). Within each tissue, values with different letters and superscripts: $p<0.05$.

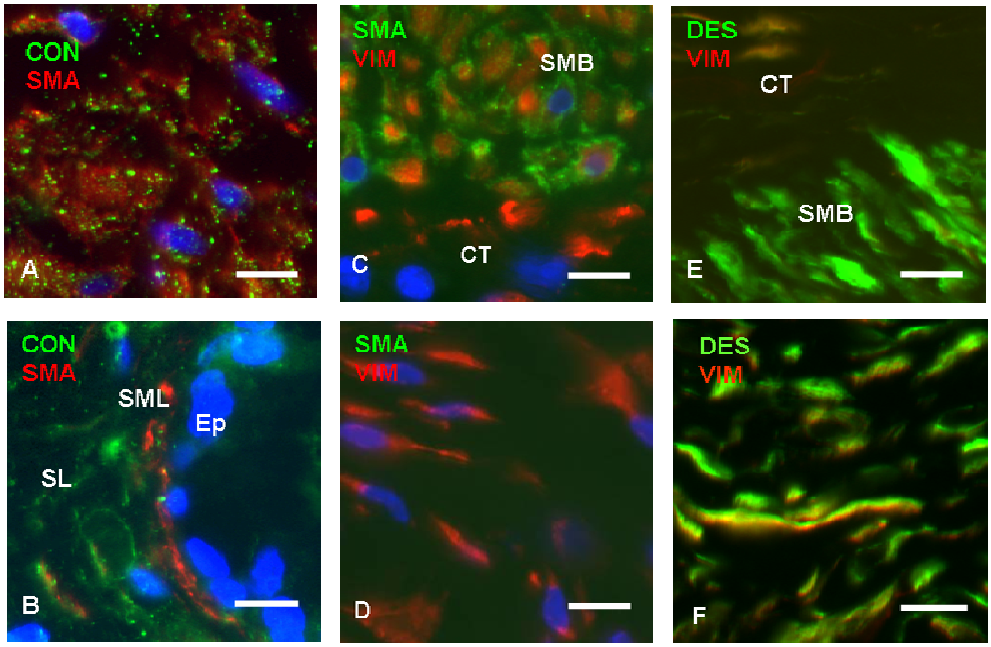

Figure 4.4 Immuno histochemical fluorescent staining of the deep (A, C and E) or superficial (B, D and F) stromal layer of the bovine cervix. A and B: Connexin-43 (green) and Smooth muscle actin α (SMA, red) double staining; C and D: SMA (green) and vimentin (red) double staining, showing the co-expression of SMA with vimentin in the muscle bundle; E and F: Desmin (green) and vimentin (red) double staining, showing the co-expression of vimentin with desmin in both superficial and deep stroma. Nuclear staining with DAPI (blue). SMB: smooth muscular bundle; CT: connective tissue; E: epithelial cells; SL: superficial stromal layer; SML: subepithelial muscular layer. Bar = 10 μm.

smooth muscle bundles differed clearly between the different biopsy time-points. The cells within the bundles were loose at 185d and 275d of pregnancy and were most dispersed at parturition (Fig. 4.1D). At that time, many cells were fragmented, or less elongated than during pregnancy. However, the smooth muscle cells remained organised in bundles. At 30 days postpartum, the cells were thinner and more closely packed together than at the other time-points (Fig. 4.1C). Within the SMA positive bundles, the SMA OD did not change with advancing pregnancy and towards parturition (Fig. 4.5B). Within the superficial layer, the vascular walls stained positive and a sub-epithelial layer of SMA positive cells was observed (Fig. 4.4B). This thin sub-epithelial layer was not continuous and showed no differences in staining intensity between the different stages. It seemed not to be associated with blood vessels as vascular lumen or erythrocytes were not observed in its proximity.

Desmin staining

Desmin staining was strong in the smooth muscle bundles, and less intense in the cells in the connective tissue between the bundles (Fig. 4.4E) and in the superficial stroma (Fig. 4.4F). The desmin OD of the smooth muscle bundles did not change between 185d and 275d of pregnancy, decreased between 275d of pregnancy and parturition ($p<0.05$), and increased again between parturition and 30 days postpartum ($p< 0.05$) (Fig. 4.5C). The desmin staining of the superficial layer was lower than that of the smooth muscle bundles and did not change in time (data not shown).

Vimentin staining

Vimentin staining was present within the smooth muscle bundles, in cells of the connective tissue between these bundles and in the superficial stromal layer (Fig. 4.4C and D). Within SMA positive cells, staining of vimentin was distributed over the whole cytoplasm, in contrast to SMA, which was present at the periphery of the cells. The vimentin OD of the smooth muscle bundles did not change between 185d pregnancy and 275d pregnancy and tended ($p=0.1$) to decrease from 275d pregnancy to parturition. The vimentin expression increased from parturition to 30d after parturition ($p<0.05$, Fig. 4.5D). The level of vimentin staining of the superficial stroma

was lower than that of the smooth muscle bundles and did not change between the different biopsy time-points.

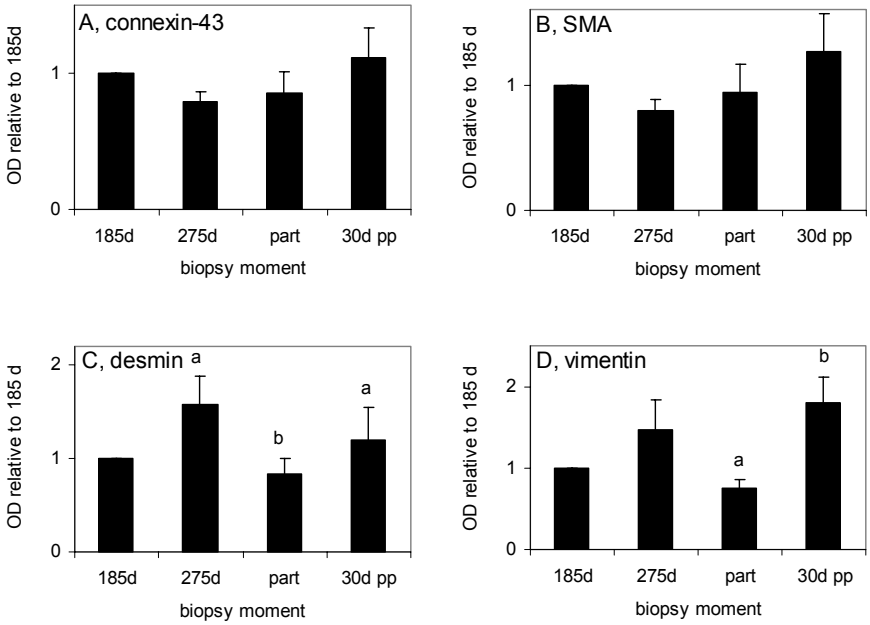

Figure 4.5 Mean staining (±SEM) of connexin-43 (A), SMA (B), desmin (C) or vimentin (D) of the stromal muscular bundles at different time points, relative to the staining at 185 d pregnancy. Values with different letters: $p<0.05$.

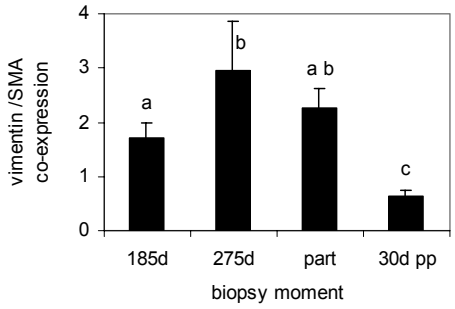

Figure 4.6 Relative amount (± SEM) of vimentin staining of SMA positive tissue compared to vimentin staining of SMA negative tissue, in bovine cervical biopsy sections that were double stained with vimentin and SMA. Serial biopsies are obtained from 5 cows at 185d of pregnancy, 275d of pregnancy, at parturition and 30d after parturition. Values with different letters: $p<0.05$.

As illustrated by Figures 4.4E and 4.4F, almost all cells that expressed vimentin also expressed desmin, both in the deep and superficial stromal layer. However, the smooth muscle bundles stained relatively more intensely for desmin, while other stromal tissue stained relatively more intensely for vimentin.

At day 185 and 275 of pregnancy and at calving, within the deep stromal layer, tissue that stained positive for SMA exhibited a higher level of vimentin staining than areas that stained negative for SMA. This is depicted in Figure 4.6, where the mean OD of vimentin staining of SMA areas divided by the OD of vimentin staining of SMA negative areas reaches values greater than 1.0. The proportion of double staining increased between day 185 and 275 of pregnancy ($p<0.05$), was lower at parturition ($p<0.05$) and had decreased further at 30 d after calving ($p< 0.05$).

Discussion

The present study shows that stromal smooth muscle cells of the bovine cervix remain organised in smooth muscle bundles during late pregnancy and at parturition and stain abundantly for SMA and connexin-43 (Fig. 4.4). Connexin-43 was localised directly surrounding the cytoplasm, suggesting the presence of gap-junctions and SMA was present in the periphery of the smooth muscle cells, indicating a contractile function [27]. These findings would indicate that the stromal smooth muscle cells are able to contract during pregnancy and parturition. The cervical extracellular matrix loses its strength at the end of pregnancy [28-30] and contraction of the smooth muscle bundles might maintain cervical closure [13] or integrity at this time.

However, factors in the cervical stroma enhancing the contractile properties of the smooth muscle bundles, such as cellular density, the abundance of SMA and connexin-43 protein, did not increase at parturition. The cellular density of the smooth muscle bundles of the bovine cervix decreased significantly with advancing pregnancy. This could have resulted from apoptosis, as has been shown in rat cervical fibroblasts [31,32] or from a relative increase in ECM volume, for instance by the synthesis of new collagen fibrils or by an increased glucosaminoglycan content [33] and

water retention [23]. Additionally, the OD of connexin-43 staining and the connexin-43 mRNA expression, which are good indicators of the amount of connexin-43 present [34], were not increased at parturition. The lack of any increase in connexin-43 mRNA and protein expression in the parturient cervix contrasts with previous reports from other species where there were significantly increasing connexin-43 mRNA and protein levels in the parturient myometrium [15,17,35]. In these studies, it was shown that the increased uterine connexin-43 level was associated with an increased contractility and that this increased expression at parturition was due to an increase in plasma oestrogen levels. Furthermore, the SMA mRNA expression, which was high during 185d and 275d pregnancy, had decreased at parturition.

During parturition, the cervix is subjected to stretch and interestingly in this respect is the finding that in cardiovascular smooth muscle cells the SMA expression decreases under influence of mechanical stretch [36-38]. Plasma oestrogen levels are high during bovine parturition [39], which places the decreased SMA mRNA expression we found in the parturient cervical stroma, in direct contrast with the finding by Hsue et al. that oestrogens stimulate SMA mRNA expression in the rat uterus [40].

However, the changes that we found in SMA mRNA expression were not reflected by parallel changes in protein expression (Fig. 4.5). It could be that SMA protein expression is post transcriptionally regulated and that mRNA expression does not reflect the actual amount of protein present. At the protein level, the SMA staining tended to decrease between days 185d and 275d of pregnancy. A decrease in SMA staining at the vaginal side of the human cervix at parturition has recently been shown using immuno histochemical staining [41] and this contrasts with a marked increase in smooth muscle content at the uterine side of the cervix [14].

Together, the combination of a low cellular density, a constant connexin-43 expression and decreased SMA expression towards parturition suggests that the bovine cervical stroma is less well prepared for coordinated contractile activity at parturition. This would correspond with our previous findings in cows that no EMG activity could be detected in this tissue during calving [4], even though contractions of the cervical stroma of non-pregnant

cows have been observed *in vitro* spontaneously [6] and in response to oxytocin [4] while cervical smooth muscle cells contain oxytocin receptors at parturition [42].

We showed that the cervical stromal smooth muscle cells expressed vimentin. This is rather uncommon for visceral smooth muscular tissue [21,43]. The cervix shares this feature with the human uterine myometrium and the cellular localisation of vimentin and SMA is the same in the cervix and uterus [44,45]. The vimentin and desmin staining of the smooth muscle bundles of the bovine cervical stroma tended to increase from day 185 to day 275 of pregnancy (Fig. 4.5) and desmin mRNA expression was high at day 275 of pregnancy (Fig. 4.2). Experiments with vimentin [46] and desmin [47,48] in knock out mice showed that both vimentin and desmin play a role in cellular contraction and maintenance of cellular integrity. Desmin might also be involved in gene regulation and the communication between the nucleus and the extra cellular matrix [49]. Therefore, the expression of vimentin and desmin might play a role in the maintenance of the cellular integrity of the cervix, which is challenged during term pregnancy, when the strength of the EMC is decreased [50,51]. Vimentin is also implicated in the regulation of secretory processes [52]. In fact, in vascular smooth muscle cells, vimentin expression is associated with MMP secretion [19] and the presentation of a secretory phenotype [18]. Therefore, the vimentin expression in cervical smooth muscle cells and the increase in co-localisation with SMA at day 275 of pregnancy (Fig 6) might indicate that these cells predominantly have a major secretory function at that time. This would support the finding that human cervical smooth muscle cells secrete MMPs which are involved in remodelling of the ECM [22,53,54]. It has previously been reported that uterine smooth muscle cells shift from a secretory into a contractile phenotype during fetal development [55] and that oestrogens stimulate the presence of a secretory phenotype in myometrial cells of adult rats [56]. Furthermore, rat vaginal smooth muscle cells shift from a contractile into a secretory phenotype with advancing pregnancy [20]. The ability of cells to alter their phenotype, also called “phenotypic modulation” [18], has been described extensively for vascular smooth muscle cells, and has also been reported for human cervical fibroblasts [50,57].

Apart from the smooth muscle cells, the bovine cervical tissue contained many fibroblasts. We showed that these fibroblasts co-express vimentin and desmin. This is in agreement with previous descriptions for rat and human cervical fibroblasts [32,50,51] and suggests that not only the smooth muscle cells but also the fibroblasts are prepared to restrain stretch during parturition.

In conclusion, our study indicates that cervical stromal smooth muscle cells are less prepared to generate coordinated contractions during late pregnancy and parturition, but exhibit characteristics of a secretory phenotype which suggests that they play a role in the metabolism of the extracellular matrix.

Acknowledgements

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7

MMP-2 expression

precedes

the final ripening process of the bovine cervix

5

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Abstract

Collagen is denatured in the gradual cervical ripening process during late pregnancy, already before the onset of final cervical ripening at parturition. Matrix Metallo Proteinases (MMPs) might be responsible for this process. To investigate the presence and potential function of MMPs at the different stages of the ripening process, serial cervical biopsies were obtained from 10 cows at days 185 and 275 of pregnancy (approximately 5 days before calving), at parturition and at 30 days after parturition. The mRNA and protein expression of MMP-1, MMP-2 and MMP-9 and of the tissue inhibitors of MMPs (TIMP)-1 and TIMP-2 were semi-quantitatively determined using RT-PCR, respectively zymography, Westernblot and ELISA techniques and the localization of MMP-2 protein and presence of granulocytes by immunohistochemistry and Luna staining. At parturition compared to 185 d pregnancy MMP-1 and MMP-9 protein expression, as well as the numbers of granulocytes, were significantly increased by 3, 9 and 26 fold respectively. MMP-2 mRNA and protein expression had already increased 2.5 ($p<0.05$) and 2 fold ($p<0.07$) at 5 days before parturition, prior to final ripening. At that time, MMP-2 was present in smooth muscle cells and extra cellular matrix. TIMP-1 mRNA expression was significantly increased at parturition and TIMP-2 mRNA expression peaked at 5 days before parturition. The increased expression of MMP-2 at 5 days before parturition, suggests that in the cow MMP-2 is responsible for collagen denaturation in the last part of gradual cervical ripening, while MMP-1 and MMP-9 are only active during the final cervical ripening process at parturition.

Introduction

The cervix is firm, due to connective tissue which contains a large quantity of extra cellular matrix (ECM) including type I and type III collagen [1,2]. The collagen fibrils are reorganized during pregnancy and subsequently degraded at parturition, to allow easy distension of the cervix [3,4]. This process, called cervical ripening, is functionally divided into two stages; 1) a stage of gradual ripening without signs of inflammation, which takes place during the third trimester of pregnancy or possibly even earlier [5,6] and prepares the cervix for 2) a stage of final ripening which occurs as late as during labor and cervical dilatation [1,7]. In the cow, this latter stage has previously been referred to as the final softening stage [8].

Matrix Metallo Proteinases (MMPs) are responsible for digestion and denaturation of the collagen fibrils in the final cervical ripening [9]. These MMPs are secreted by stromal cells [10] and by granulocytes that have invaded the cervix in this very last stage of pregnancy [11]. MMPs differ with regard to their regulation, and substrate specificity [12-14] which implies that different MMPs have different functions in the process of cervical ripening. The role of MMP-2 and MMP-9 in final cervical ripening has been demonstrated in humans, rodents and rabbits [10,15-17]. MMP-1 might also be involved in final cervical ripening as has been shown in human, rodent and rabbit cervical tissue [7,18,19] although other studies failed to detect MMP-1 in the cervix of parturient women *in vivo* [20-22]. MMPs, once secreted, are inhibited by Tissue Inhibitors of Matrix Metallo Proteinases (TIMPs). In the human early post partum cervix TIMP-1 and TIMP-2 have been detected [1,23]. These factors may inhibit MMP activity in the cervix if they are also present in the ECM during cervical ripening.

Extensive remodelling of the cervical tissue already takes place during gradual cervical ripening [1,24]. During this process, changes in the proteoglycan content and increased water retention are observed, as well as denaturation of collagen fibrils, that become more soluble as is shown for cows and humans [8,25]. The presence of denatured collagen during the gradual ripening process suggests MMP activity [8]. However,

according to Word et al. (2007), the involvement of MMP in the process of gradual cervical ripening remains to be elucidated.

The bovine cervix has proven to be a sound model to study the process of gradual cervical ripening [26], because it is possible to take biopsies from the same animal repeatedly during pregnancy and parturition. Therefore, this approach was used to determine MMP-1, -2 and -9 expressions, together with TIMP-1 and -2, within the bovine cervical tissue during both gradual and final cervical ripening, at day 275 of pregnancy and at parturition respectively. Since MMP-1 and MMP-2 have collagenase activity, while MMP-9 only digests denatured collagen type I [13], it was expected that MMP-1 and/or MMP-2 would be expressed during gradual cervical ripening before the invasion of leukocytes into the tissue, while MMP-9 would be present predominantly at final cervical ripening.

Materials and methods

Experimental design

Cervical biopsies were collected from 10 Holstein Friesian cows which belonged to a commercially kept, high yielding dairy herd housed at the experimental farm of the Faculty of Veterinary Medicine, Utrecht University. The cows were fed according to their individual nutritional needs as defined by their level of milk production and stage of gestation. All cows delivered a healthy calf without signs of dystocia. The experimental procedure was approved by the Ethical Committee of the Veterinary Faculty of Utrecht University. Cervical biopsies were obtained trans-vaginally, using a skin biopsy punch of 6 mm diameter (Kai industries co. Ltd. Oyana, Japan) as previously described [8]. The cervical tissue samples included the superficial- and the deep stromal layers of the cervix. Tissue sampling was performed at day 185 (± 3) and day 275 (± 1) of gestation, at parturition on average 2.1 (± 2) hours after spontaneous calving but before delivery of the placenta, and at 30 (± 3) days after parturition. The biopsies were cleaned of blood and mucus and cleaved lengthwise. One part of each biopsy was snap frozen in liquid nitrogen, and stored at -80 °C until further analysis. The other part of each biopsy was fixed in 4.5 % formaldehyde in phosphate buffered saline (PBS) for 24 h and embedded in paraffin. Due to

the limited size of the biopsies, there was not enough material to perform all analyses with the biopsies of ten cows. Numbers of animals from which samples were used for each analysis are indicated in the appropriate paragraph. A series of samples from one cow was always processed and analyzed within one session.

RT-PCR for MMP-1, -2 and -9 and TIMP-1 and -2

The -80 ºC frozen tissue samples were pulverized with a mortar and pestle. Half of the sample was then used for RNA extraction and the other half for protein extraction. RNA was isolated using RNeasy Fibrous Tissue Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. In short, the pulverized sample was transferred to 300 µl lysis buffer, drawn through a 20 gauge needle, to resuspend remaining clumps, and subjected to a proteinase K treatment (10 mAU/ml) at 55 ºC for 10 min, followed by centrifugation at 1000 g for 3 min. The supernatant was run through a Qiagen mini column, after which the column was washed twice. RNA was eluted from the spin column with 50 µl RNAse free water, quantified by spectrophotometry (260 nm), controlled for quality on a 1 % agarose gel and stored at –80 C until further use.

Complementary DNA was synthesized using the iScript™ cDNA Synthesis kit (Biorad, Luxembourg) according to the instructions of the manufacturer. Briefly, 5 µg RNA was added to 4 µl iScript Reaction Mix (Biorad, Luxembourg), 1 µl iScript Reverse transcriptase (Biorad, Luxembourg) and RNase free water to a total volume of 20 µl. The complete reaction mix was incubated in a thermalcycler (Mycycler, Biorad, Luxembourg) at 25 ºC for 5 min and at 42 ºC for 30 min after which the enzymes were inactivated at 85 ºC for 5 min. Samples were diluted to 10 ng/ml working solutions and stored at 4 ºC.

Real time PCR was performed using a MyiQ system; iCycler and detection unit (Biorad, Luxembourg) using SYBR-Green Supermix (Biorad, Luxembourg); cDNA input was 50 ng and the concentration of the forward and reverse primers was 330 nM. Sequences of the different forward- and reverse primers are shown in Table 5.1. Product specificities of the primer sets were verified by melting curve analysis after termination of the PCR.

	primer sequence 5'- -3' primer sequence 3'- -5'
GAPDH (NM_001034034)	F: AAG TTC AAC GGC ACA GTC AAG G R: CAT ACT CAG CAC CAG CAT CAC C
MMP-1 (NM_174112)	F: TTG ATG CCA TAA CTA CAA TTC G R: TCA TAA GCA GCT TGA AGT CC
MMP-2 (NM_174745)	F: CAA TAC CTA AAC ACC TTC TAC G R: TTC CGC ATG GTC TCA ATG
MMP-9 (NM_174744)	F: TGT TAG AGA GCA CGG AGA TGG R: CGC CTT TGC CCA GAG ACC
TIMP-1 (NM_174471)	F: GGG ATT CAC CAA GAC CTA TGC R: AGC TGG TCC GTC CAC AAG
TIMP-2 (NM_174472)	F: GGC AAC GAC ATC TAC GGC AAC C R: GAC CCC ACA CAC GGC AGA GG

Table 5.1 Accession numbers and sequences from the primers that were used for the RT-PCR analysis of bovine cervical tissue samples (F: forward primer; R: reverse primer).

For each sample, duplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model for the relative expression ratio in real-time PCR, the $2^{-\Delta\Delta Ct}$ –method [27,28]. An earlier study on the cervix of the pregnant and parturient cows showed that GAPDH could be regarded as household gene because its expression was highly correlated (r = 0.9) with that of β- actin [29].

Zymography for MMP-2 and -9 activities

Zymography was performed according to the method described by Creemers et al (1998). Pulverized tissue was homogenized in 100 µl lysis buffer (70 mM Hepes (pH 7.4); 142 mM NaCl; 10 mM $CaCl_2$; 5 % Glycerol; 1% Triton X-100; 1.5 mM NaN_3), freeze-thawed 3 times using liquid nitrogen after which they were incubated overnight at 4 ºC under agitation. After centrifugation (10,000 rpm; 4 ºC; 15 min), the supernatant was collected and protein concentrations were determined using the bicinchinic

acid method (BCA; Pierce, Rockford, USA), with BSA as a standard. The samples were diluted in lysisbuffer and mixed 1:1 with sample buffer (0.125 mM TRIS (pH 6.8); 4% sodium dodecylsulfate; 20 % glycerol; 10 mM Na EDTA; 0.0025 % Bromophenol blue) to a final amount of 20 μg protein per lane. The samples were subjected to electrophoresis in SDS polyacrylamide gel containing 3 mg/ml gelatin. After washing the gels in 1% Triton X-100 to remove the SDS, the gels were incubated at 37 ºC over-night in a buffer containing 50 mM TRIS (pH 7.5); 1% Triton X-100; 5 mM $CaCl_2$ and 3 mM NaN_3. The gels were then stained with Coomassie Brilliant Blue G250 and destained in a solution consisting of 5 % methanol and 6 % acetic acid. Areas of gelatinolysis appeared as clear bands against a blue background. The gels were photographed (Olympus C-4000 zoom) after which the rate of gelatinolysis was semi-quantitatively analyzed with AlphaEaseFC™ software (version 3.2.3; Alpha Innotech; San Leandro; USA). Based on their molecular weights, the MMP activities were attributed to the MMP-2 (72 kDa) or MMP-9 (92 kDa). In some cases both enzyme and pro-enzyme appeared to show activity in the gel. In those cases, the optical densities of these two bands were added together.

Western blot analysis for MMP-1 and TIMP-1

MMP-1 and TIMP-1 protein expressions were determined by performing densitometry after electrophoresis and Western blotting.

From each sample, as prepared for the zymography procedure, 40 μg protein was subjected to SDS polyacrylamide gel electrophoresis and blotted to a nitrocellulose membrane. The membrane was incubated overnight with Rabbit Anti- Human Matrix Metalloproteinase-1 hinge region polyclonal antibodies (Biomol; USA) with cross reactivity for the bovine latent and active form of MMP-1, or incubated for 1.5 h with Mouse Anti-Bovine TIMP-1 Monoclonal Antibodies (MAB3300, Chemicon, USA). The membranes were subsequently washed and incubated for 1 h with peroxidase-conjugated Goat Anti- Rabbit or Goat Anti-Mouse antibody (Pierce, IL). The protein bands were visualized with chemilumines-

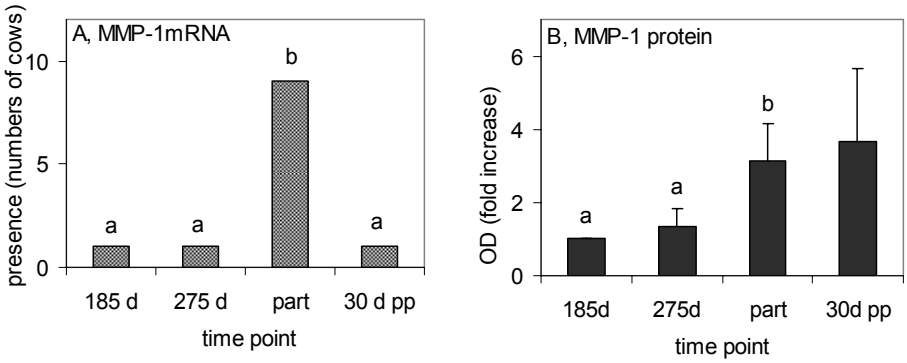

Figure 5.1 (A) Numbers of cows (out of 10) in which MMP-1 mRNA expression could be determined at different time-points (RT-PCR) and (B) Optical densitometry of Westernblot analysis (n=7) in cervical biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). (a-b): significant difference ($p<0.05$).

cence using the Pierce supersignal system (Pierce, IL) and photographed. Tubulin staining (mouse monoclonal B-5-1-2, Sigma, CA) was used as a control for equal loading. The amount of chemiluminescence was semi-quantitatively determined with AlphaEaseFC^{TM} software and calculated, by dividing the amount of chemiluminescence of MMP-1 or TIMP-1 by that of tubulin.

ELISA for TIMP-2

The TIMP-2 protein concentration was determined with an ELISA test (Amersham TIMP-2 human biotrak ELISA system, Amersham, UK). Cervical samples, as prepared for the zymography, containing 40 µg protein in 50 µl solution were added to the wells in duplicate and further procedures were carried out according to the manufacturer's recommendations. Serial dilutions of a standard TIMP-2 solution were used as a reference for the quantification of TIMP-2 in the biopsy protein extracts.

Histology

Tissue sections from all 10 cows, 5 μm thick, were stained with the Luna staining [30] to determine the presence of granulocytes. Analysis of the biopsies was performed by light microscopy using a 40 X objective. The number of granulocytes was counted in ten fields (0.096 mm^2) of the superficial-, and ten of the deep stromal layer. For each sample, the mean number of granulocytes per mm^2 was calculated over these twenty fields.

Immuno histochemistry for MMP-2

Since in the course of the experiment it appeared that MMP-2 activity was high at 275 days of pregnancy, while the number of granulocytes was low, immuno-histochemistry was performed to identify the stromal cells, which produced MMP-2. Five μm thick tissue sections were obtained from the paraffin embedded biopsies from 5 cows and mounted on a coated glass slide (Superfrost plus, Erie Sc. Co.,USA). The sections were deparaffinized and rehydrated with a xylene and ethanol series and rinsed three times with PBS between the following steps. The sections were treated with 3% H_2O_2 in PBS for 30 min and incubated for 30 min with normal goat serum (NGS, 1:10) in PBS to block non-specific binding of the antibodies. Subsequently, the sections were incubated overnight at 4 °C with Rabbit Anti- Human MMP-2 polyclonal antibodies, which cross reacts with Bovine MMP-2 (Chemicon, USA) 1:50. Finally, they were incubated for 45 min with Goat Anti- Rabbit Envision (DAKO, Denmark), incubated for 7 min with DAB (DAKO, Denmark) rinsed three times with MilliQ water, counterstained with hematoxylin for 2 min, rinsed with tapwater, dehydrated and mounted with Eukitt (EMS, UK). Control sections were incubated with the secondary antibodies only. The localization of the brown MMP-2 staining was assessed by light microscopy by comparing the intensity of the staining of the ECM with that of the stromal cells.

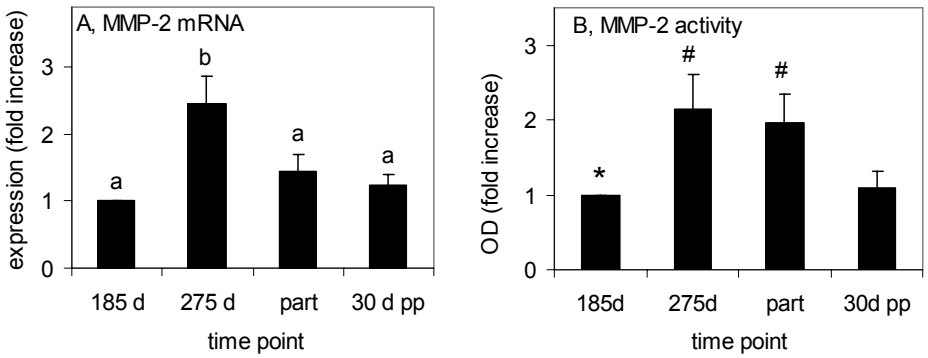

Figure 5.2 (A) Relative MMP-2 mRNA expression (RT-PCR, n =10) and (B) Gelatinase activity (n=8) in bovine cervical biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). (*- #): tendency to significance p<0.07; (a-b): significant difference (p< 0.05).

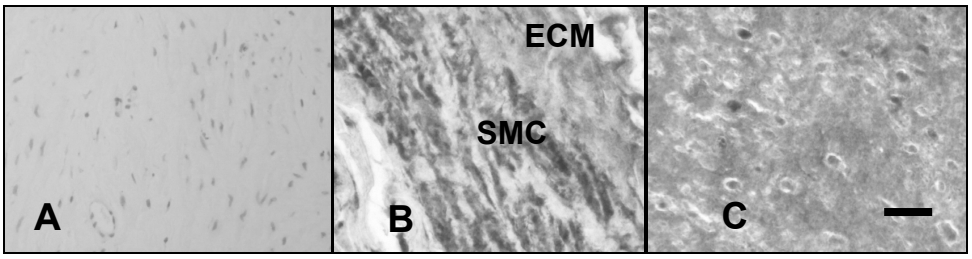

Figure 5.3 Immunohistochemical staining (DAB) for MMP-2 in the deep stromal layer of the bovine cervix. (A) Control without primary antibody; (B) 275 days of pregnancy. Strong staining for MMP-2 was present in the cytoplasm of the smooth muscle cells (SMC) and less in the extra cellular matrix (ECM). (C) At parturition; MMP-2 staining in the cytoplasm and in the ECM was equally strong. However, the perinuclear areas of the cells are not stained. Bar: 50 μm.

Statistical analysis

Changes in mRNA or protein expression between biopsies that were taken at the different stages around calving were statistically analyzed with the repeated measures method in SPSS (version 12.0.1, SPSS Inc., USA).

When a significant time effect was present, the contrasts between the different time-points were further analyzed with a paired t-test. When a significant time effect was absent, although with prominent differences between two or more time-points, a paired t-test was used with Bonferonni correction. Differences were regarded as statistically significant when $p<0.05$. For use in the Figures, the values at the different time-points were expressed relative to the value at 185d pregnancy, which was given as 1.0.

Results

MMP-1

The MMP-1 mRNA expression was detectable in one cow at 185 days of pregnancy and at 30 days after parturition and in another cow at 275 days of pregnancy (Fig. 5.1A). At calving, MMP-1 mRNA was expressed in 9 of the10 cows. The probability of this difference in MMP-1 mRNA expression is $9*10^{-9}$, which is highly significant. The MMP-1 protein expression remained at the same level between 185 and 275 days of pregnancy (Fig. 5.1B). It was increased three fold at calving ($p< 0.05$) compared to 185d of pregnancy and remained high at 30d after calving.

MMP-2

The MMP-2 mRNA expression (Fig. 5.2A) increased 2.5 fold between 185d and 275d of pregnancy ($p< 0.05$), after which it returned to about the same level as it was at 185d of pregnancy. MMP-2 gelatinase activity increased two fold but not significantly between 185d and 275d of pregnancy ($p< 0.07$) and remained high at calving after which it returned to a similar level as at 185d of pregnancy (Fig. 5.2B). At 275d of pregnancy both stromal cells (fibroblasts and smooth muscle bundles) and ECM stained for MMP-2, while staining intensity was more abundant in the cytoplasm compared to the ECM (Fig. 5.3B). At calving however, staining of the stromal cells was reduced, compared to that of the ECM, and the peri-nuclear areas of the cells were even totally depleted of MMP-2 in some cows (Fig. 5.3C).

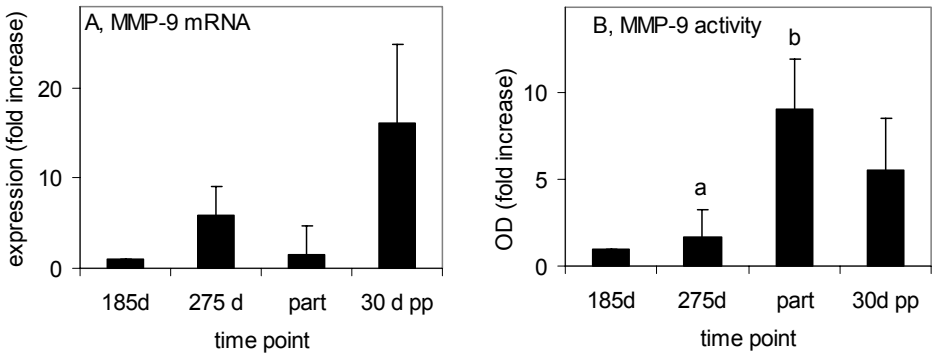

Figure 5.4 (A) Relative MMP-9 mRNA expression (RT-PCR, n=10) and (B) Gelatinase activity (n=6) in bovine cervical biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). (a-b): significant difference ($p<0.05$).

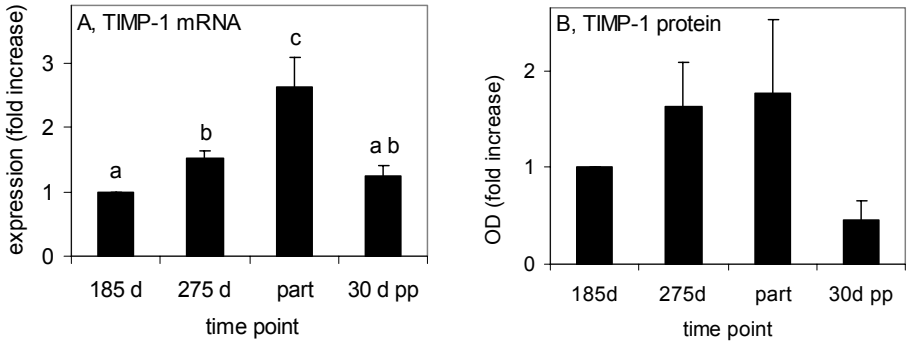

Figure 5.5 (A) Relative TIMP-1 RNA expression (n=7) and (B) Optical densitometry of Western blot analysis (n=4) in bovine cervical biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). Different letters indicate significant differences ($p<0.05$).

MMP-9

The mean MMP-9 mRNA expression patterns differed largely between the cows and no significant differences between the time-points were found (Fig. 5.4A). In six cows MMP-9 gelatinase activity was very low at 185d and 275d of pregnancy, 9 fold increased at calving ($p< 0.05$) and remained high at 30d afterwards (Fig. 5.4B). In two cows, no MMP-9 enzyme activity could be determined at any of the biopsy time-points.

TIMP-1

The TIMP-1 mRNA expression increased 1.5 fold from 185d to 275d of pregnancy ($p<0.05$) and further, to 2.6 fold at calving ($p<0.05$) after which it returned to a similar level as at day 185 of pregnancy ($p<0.05$, Fig. 5.5A). TIMP-1 protein expression in the cervical samples was undetectable in 3 of 7 cows. In the remaining 4 cows, the average protein expression followed the same pattern as the mRNA expression (Fig. 5.5B) but the differences between the biopsy time-points were not significant.

TIMP-2

The TIMP-2 mRNA expression tended to increase 1.5 fold from185d to 275d of pregnancy after which it returned to a similar level as at day 185 of pregnancy ($p< 0.05$, Fig. 5.6A). The average TIMP-2 protein level in the cervical samples followed the same pattern, over the successive time-points, as the mRNA expression but the differences between the biopsy time-points were not statistically significant (Fig. 5.6B).

Granulocyte density

The histochemical staining showed that granulocyte density at 180d and 275d of pregnancy and 30d after calving was on average 3.5 cells/ mm^2. Compared to these time-points the granulocyte density increased 26 fold ($p<0.05$) at parturition (Fig. 5.7). The granulocytes were present in both the superficial and the deep layer of the cervical stroma.

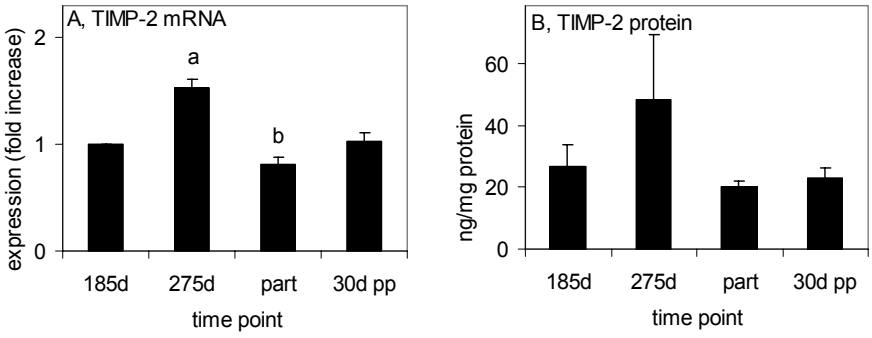

Figure 5.6 (A) Relative TIMP-2 mRNA expression (RT-PCR, n=7) and (B) Protein concentration (ng/mg total protein, n=6), determined using ELISA, in bovine cervical biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). (a-b): significant difference ($p<0.05$).

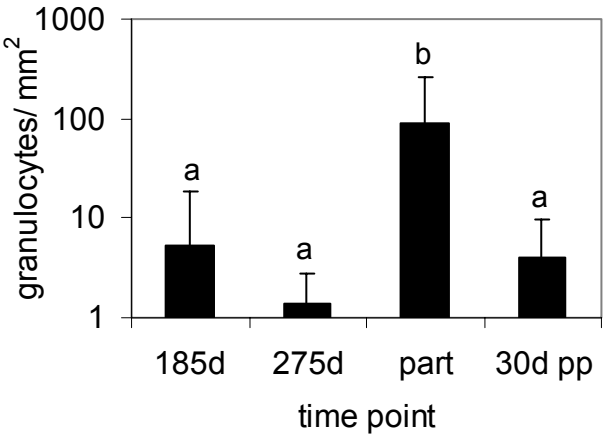

Figure 5.7 Mean granulocyte density (log scale, n=10) of the bovine cervical stroma (including both superficial and deep layers) in biopsies obtained at 185 and 275 days of pregnancy; at parturition (part) and 30 days post partum (30d pp). (a-b): significant difference ($p<0.05$).

Discussion

MMP-2 mRNA expression and enzyme activity in the bovine cervix were already maximal at day 275 of pregnancy, in contrast to MMP-1 and MMP-9 expression. At that time-point, which was on average 5 days before parturition, MMP-2 was present in smooth muscle cells and the surrounding ECM (Fig. 5.3B), while final ripening had not yet started, as indicated by the near absence of granulocytes. This early presence of MMP-2, which has also been demonstrated in humans [10], indicates that MMP-2 secreted by smooth muscle cells may cause denaturation of the collagen fibrils during the gradual cervical ripening process. Thus expression of MMP-2 mRNA does not require a steep fall in plasma progesterone levels (such as occurs 2-3 days before calving), or a rise in cervical pro-inflammatory cytokine levels, that occurs during the process of cervical dilatation [5,11,31]. This finding in cattle is supported by earlier reports that the MMP-2 level, in contrast to MMP-1 and MMP-9, is not augmented after withdrawal of progesterone in rabbit cervical tissue *in vitro* or after *in vivo* treatment of women with progesterone antagonists [17,32,33]. It is also in agreement with the observation that MMP-2 secretion is not stimulated by pro-inflammatory cytokines *in vitro* in contrast to MMP-1 and -9 [18].

The data presented here demonstrate that granulocytes were abundantly present in the cervix at parturition, but that MMP-2 mRNA expression had decreased, while MMP-2 protein presence in the ECM remained high (Fig. 5.3C). Pro-MMP-2 is stored in cytoplasmic vesicles and in response to an appropriate signal [34-36] like inflammatory mediators, it seems that rapid secretion, rather than pro-MMP-2 transcription is stimulated [16]. Once secreted into the ECM, pro-MMP-2 becomes activated on the cell surface by Membrane Type MMPs (MT-MMPs), which require TIMP-2 [37,38]. MT-MMP binds TIMP-2, and this complex binds proMMP-2, which is then converted to an active form of MMP-2. However, when tissue levels of TIMP-2 become sufficiently high, it may also inhibit MMP-2 activity by forming a stable TIMP-2-MMP-2 complex. In this study, increased levels of TIMP-2 mRNA were measured at day 275 of pregnancy but the TIMP-2 protein levels did not change significantly over time. Therefore, the role of TIMP-2 during cervical dilatation remains to be determined.

To the best of our knowledge, the present study is the first to describe the increased MMP-1 mRNA and protein expression at term parturition in a semi quantitative way. MMP-1 mRNA expression was only detectable during parturition, simultaneously with increased MMP-1 and MMP-9 protein levels and the presence of high numbers of granulocytes. This finding corresponds to previous observations in experiments with human cervical cells *in vitro*, that MMP-1 and MMP-9 mRNA expression increased in response to pro- inflammatory stimuli or mechanical stretch [18,39]. The increased MMP-1 and MMP-9 presence at parturition, as found in the present study, may have resulted from an inflammatory cascade, evoked by the granulocytes, stimulating production by stromal cells or produced by the granulocytes themselves. These MMPs might cooperate with MMP-2 in the final digestion of the collagen fibrils [20].

Binding of TIMP-1 could have dampened the MMP-1 activity at parturition, because TIMP-1 levels were increased at that time which corresponds with previous descriptions in term pregnant rats [16] and parturient woman [9]. Whether TIMP-1 actually inhibits the MMP-1 activity at parturition is not addressed by the present study, because the exact protein quantities and histological localization of TIMP-1 could not be determined. It has been suggested that TIMP-1 and TIMP-2 are available at parturition to inactivate MMP activity immediately after expulsion [1]. However, in the present study, TIMP-1 was already present at 185 d pregnancy, which is consistent with a previous immuno histochemical study in humans [20]. This might indicate that TIMP-1 is also involved in the regulation of the MMP activity before parturition.

In summary, the present study indicates that MMP-2 which is produced by smooth muscle cells, plays a role in the gradual ripening process at the end of pregnancy, whereas MMP-1 and MMP-9 are present during parturition and, thus, are associated with the final ripening process. It is indicated that TIMP-1 and TIMP-2 are present during parturition, but their regulatory role remains to be established.

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7

Cervical ripening and parturition in the cow are driven by a cascade of pro-inflammatory cytokines

6

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Abstract

The final stages of cervical ripening and parturition resemble an inflammatory process. Whereas the role of cytokines in both spontaneous and experimentally induced parturitions has been described in several small laboratory animals and humans, the involvement of pro-inflammatory and regulatory cytokines in physiologic parturition in cows has not been determined. In the present study the cytokine expression profiles were assessed in bovine cervical tissue at several stages of pregnancy and at parturition. Serial biopsies of the cervix were obtained from 10 cows at day 185 and day 275 of pregnancy (which was on average 5.4 days before parturition), at parturition and at 30 days postpartum. mRNA expression levels of IL-1β, IL-6, IL-8 IL-10 and TNFα were determined using RT-PCR and the number of neutrophils and eosinophils was estimated by Luna and Sirius Red staining. At parturition, IL-8 expression had increased 430 fold ($p<0.001$) compared to day 185 of pregnancy, large numbers of neutrophils had invaded the cervix while eosinophils remained scarce, IL-1β had increased 8 fold ($p<0.05$) and IL-6 was not significantly changed. Additionally, IL-10 was increased 10 fold ($p<0.001$) and TNFα was decreased 57% ($p<0.05$) compared to day 185 of pregnancy The large increase in expression of IL-8, enabling the influx of neutrophils, is indicative of its important role in the final stage of cervical ripening and at parturition. As previous studies have shown that neutrophils excrete MMPs, this might contribute to softening of the cervix. In contrast, the only slightly increased levels of IL1, steady concentrations of IL-6 and decreased TNFα, which potentially were the consequences of increased IL-10 expression, indicate that final ripening at term parturition is a non-infectious inflammatory process influenced by regulatory cytokines.

Introduction

Collagen is degraded before and during labor as part of the cervical ripening process which is functionally divided into two stages; 1) the gradual ripening stage taking place during the third trimester of pregnancy ,or possibly even earlier, without signs of inflammation [1,2] and 2) the final ripening which occurs as late as during labor and cervical dilatation [3,4]. The stage of final ripening is driven by an inflammatory reaction [5,6], characterized by the presence of pro-inflammatory cytokines and granulocytes, such as neutrophils and eosinophils [7-11].

For laboratory animals and humans, the involvement of IL-1, IL-6 and IL-8 in final cervical ripening has been described [12,13] but for farm animals, their involvement in this process has not been determined so far. A role for TNFα in human final cervical ripening is indicated by the findings that TNFα in cervical smooth muscle cell cultures stimulated the production of matrix degrading proteins, and that TNFα was present in the cervical tissue during pregnancy as shown by immuno-histochemical staining [14,15]. However, the involvement of TNFα in final ripening of the cervix at normal parturition remains uncertain [16], since TNFα levels remained unchanged in human term and preterm parturitions without infection [8,17-19].

In different species, the inflammatory cascade which leads to final cervical ripening shows a similar sequence of events, including granulocyte invasion, cytokine expression and collagen degradation closely before and during parturition. This implies that cervical ripening is a precisely orchestrated process, and apart from pro-inflammatory cytokines also regulatory cytokines, such as IL-10 may play a role [20]. It has been shown for human decidua and fetal membranes that IL-10 levels [21] are high during pregnancy and decreased at parturition [22]. Additionally, IL-10 reduced LPS (Lipopolysaccharide) induced uterine contractions in monkeys [23] and preterm births in rats [24]. However, the IL-10 expression in the cervix during pregnancy and parturition has not been described in any species until now. The present study aimed to elucidate the role of TNFα and other pro-inflammatory cytokines, of IL-10 and that of granulocytes at several time points of pregnancy and parturition.

The cow has shown to be an appropriate *in-vivo* model to study cervical ripening at term parturition [25]. Serial cervical biopsies are easily obtainable from different sites of the bovine caudal cervix during pregnancy and at parturition. Additionally, the rate of cervical dilatation, collagen metabolism and the expression patterns of matrix degrading proteins such as several Matrix Metallo Proteinases (MMP) and inducible nitric oxide synthase (iNOS) have already been documented for the cervix of pregnant and parturient cows [25-28]. Thus, in the present study, the kinetics of production of (pro-inflammatory) cytokines and the influx of inflammatory cells over the course of time during bovine pregnancy and parturition were investigated.

Materials and methods

Cervical biopsies were collected from 10 Holstein Friesian cows which were part of a commercially kept, high yielding dairy herd housed at the experimental farm of the Faculty of Veterinary Medicine, Utrecht University. A dry period of 8 weeks before the expected day of parturition was a standard procedure at the farm. The cows were fed according to their individual nutritional needs as defined by their level of milk production and stage of gestation. The following experimental procedure was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University. Cervical biopsies were obtained trans-vaginally, using a skin biopsy punch of 6 mm diameter (Kai industries co. Ltd. Oyana, Japan) as previously described [25]. The cervical tissue samples included the superficial- and the deep stromal layer of the caudal part of the cervix and were taken at days 185 (± 3) and 275 (± 1) of gestation, at parturition within 2.1 (± 2) hours after spontaneous calving but before delivery of the placenta, and at 30 (± 3) days after calving. The biopsies were cleaned of blood and mucus and cleaved lengthwise. One part of each biopsy was snap frozen in liquid nitrogen, and stored at -80 °C until further analysis and the other part was fixed in 4.5 % formaldehyde in phosphate buffered saline (PBS) for 24 h and embedded in paraffin. The biopsy samples had also been used for a previous study [29]. Therefore, in this study, sample size was not enough to allow all analyses for each of the cows. The numbers of cows, used for each analysis will be indicated below for each cytokine.

Histochemistry

5 μm thick sections, containing both the superficial and deep layer of the cervix, were mounted on a glass slide, deparaffinized and rehydrated with xylene and a series of alcohol and subjected to both the Luna's staining [30,31] and the Sirius Red staining [32]. For the Luna staining, the sections were incubated for 5 min in 0.9 volume of Weigert's iron hematoxylin (0.005% acid hematoxylin and 0.6% ferric chloride in 2% HCl) with 0.1 volume of 1% Biebrich scarlet. After differentiation in 1% acid alcohol, slides were washed in water. Final color development was performed in 0.5% lithium carbonate. For Sirius Red staining, the sections were incubated for 60 min in a solution containing 50 mg/l Sirius Red (Direct Red 80, Aldrich, USA), washed in water and counterstained with hematoxylin. For each time-point, the number of neutrophils and eosinophils were assessed in ten randomly distributed fields of 0.096 mm^2 with a light microscope using a 40 x objective in both superficial and deep cervical stromal layers. Neutrophils were counted in Luna stained sections and eosinophils were counted in both Luna and Sirius Red stained sections. The granulocyte density was expressed as numbers of cells per mm^2. Intestinal tissues obtained from cows with confirmed eosinophilic enteritis and paratuberculosis respectively served as controls for eosinophil staining. Nasal tissue obtained from a cow with confirmed IBR infection served as a control for neutrophil staining.

RT-PCR

The -80 ºC frozen tissue samples were pulverized with a mortar and pestle. RNA was isolated using RNeasy Fibrous Tissue Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. In short, the pulverized sample was transferred to 300 μl lysis buffer, drawn through a 20 gauge needle to resuspend remaining clumps, and subjected to a proteinase K treatment (10 mAU/ml) at 55 ºC for 10 min, followed by centrifugation at 1,000 g for 3 min. The supernatant was run on a Qiagen mini column, after which the column was washed twice. RNA was eluted from the spin column with 50 μl RNAse free water, quantified by spectrophotometry (260 nm), controlled for quality on a 1 % agarose gel and stored at –80 ºC until further use.

cDNA was synthesized using the iScript™ cDNA Synthesis kit (Biorad, Luxembourg) according to instructions of the manufacturer. Briefly, 5 μg RNA was added to 4 μl iScript Reaction Mix (Biorad, Luxembourg), 1 μl iScript Reverse transcriptase (Biorad, Luxembourg) and RNase free water to a total volume of 20 μl. The complete reaction mix was incubated in a thermalcycler (Mycycler, Biorad, Luxembourg) at 25 ºC for 5 min and at 42 ºC for 30 min after which the enzymes were inactivated at 85 ºC for 5 min. Samples were diluted to 10 ng/ml working solutions and stored at 4 ºC.

Real time PCR was performed using a MyiQ system; iCycler and detection unit (Biorad, Luxembourg) using SYBR-Green Supermix (Biorad, Luxembourg); cDNA input was 50 ng and the concentration of the forward and reverse primers was 330 nM. Sequences of the different forward- and reverse primers are shown in Table 6.1. Product specificities of the primer sets were verified by melting curve analysis when the PCR had finished.

	Forward primer sequence 5'- -3' Reverse primer sequence 5'- -3'
GAPDH NM_001034034	AAG TTC AAC GGC ACA GTC AAG G CAT ACT CAG CAC CAG CAT CAC C
Il-1β NM_174093	ACC CTC TCT CCC TAA AGA AAG C GGC ATG GAT CAG ACA ACA GTG
IL-6 NM_173923	TCA AAC GAG TGG GTA AAG AAC G CTG ACC AGA GGA GGG AAT GC
IL-8 NM_173925	CAA TGG AAA CGA GGT CTG C TTC ATT GGC ATC TTT ACT GAG G
IL-10 U00799	TGA CAT CAA GGA GCA CGT GAA TCT CCA CCG CCT TGC TCT T
TNFα NM_173966	CAT CTA CTC ACA GGT CCT C CTC TTG ATG GCA GAC AGG

Table 6.1 Sequences and accession numbers of the primers that were used for RT-PCR analysis of the bovine cervical biopsies

For each sample, duplicate Ct values were obtained and averaged. Quantification was performed using a mathematical model for the relative expression ratio in real-time PCR, the $2^{-\Delta\Delta Ct}$-method [33,34]. An earlier study carried out by our group using cervical tissue from pregnant and parturient cows showed that GAPDH could be regarded as a household gene because its expression was highly correlated (r = 0.9) with that of β–actin [27].

The data were log transformed to obtain a normal distribution and the differences in mRNA expression between successive biopsies (day 185, day 275 and parturition) in cows were statistically analyzed with the repeated measures method in SPSS (version 12.0.1, SPSS Inc., USA). Since we were primarily interested in the patterns of cytokines as involved over time during pregnancy and parturition, the data obtained at 30 days after parturition were not included in the statistical analysis. In case a significant time effect was observed using the repeated measures method, the differences between the individual time-points were further analyzed using paired t-tests. In the absence of a significant time effect but with apparent differences between the expression levels at the different time-points, a paired t-test was used with Bonferonni correction. Differences were regarded as statistically significant when $p<0.05$. The mRNA expression levels at different time points were illustrated relative to the level of mRNA expression at day 185 of pregnancy, which was normalized to 1.0.

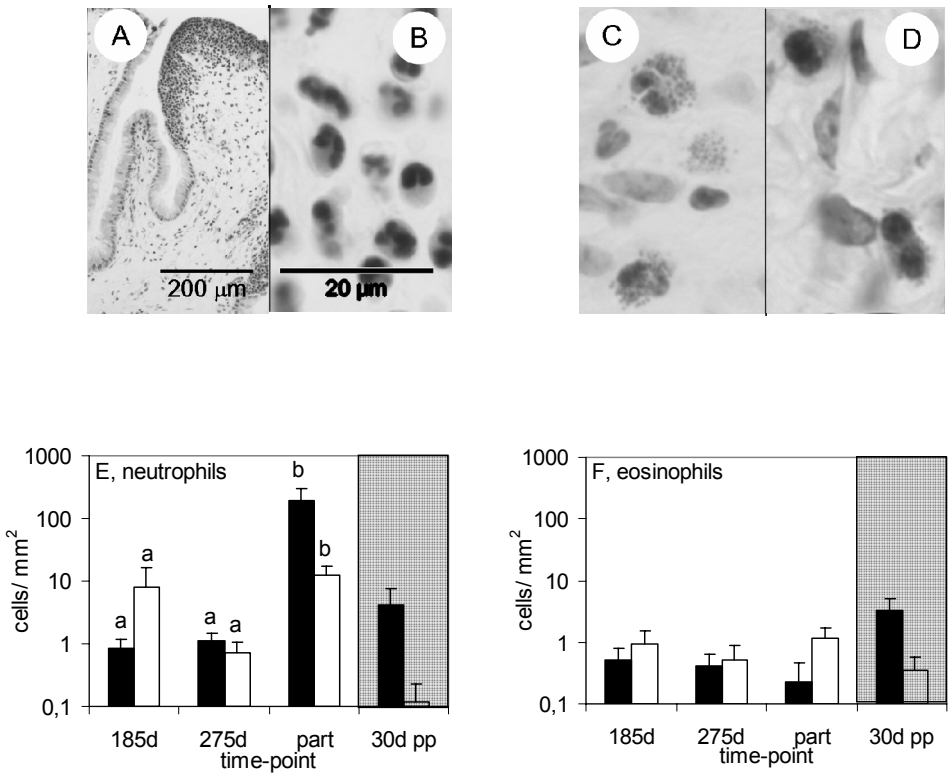

Figure 6.1 Bovine cervical tissue stained for the presence of granulocytes. A) At parturition cervical tissue shows a large invasion of neutrophils (Luna staining, X 100). B) A detailed view of the neutrophils that invaded the cervix (Luna staining, X 400). C) A detailed view of the eosinophils within the superficial layer of the bovine cervix (Luna staining, X 400). D) A detailed view of the eosinophils within the cervix, using (Sirius Red staining, X 400). E) Numbers of neutrophils and F) eosinophils per mm^2 in the superficial (■) and deep (□) stromal layers of the cervix of the cow. Cervical biopsies were obtained at days 185 and 275 of pregnancy, at parturition and 30 days after parturition. For each tissue, values with different letters are significantly different.

Results

The cows calved spontaneously and without complication at day 280 of gestation on average, which was 5.4 (±1.4) d after the biopsy at day 275 of gestation had been obtained.

Granulocyte invasion in the cervix

In the Luna staining and the Sirius Red staining, eosinophils were characterized by red intra-cytoplasmic granules and bi-lobed nuclei and could easily be distinguished from neutrophils (Fig 6.1B-D). Neutrophils were smaller and had multi-lobed nuclei and a cytoplasm that remained unstained by the Sirius Red staining but was homogeneously pale pink-orange stained by the Luna staining. As Figure 6.1E shows for all cows, the number of neutrophils was low in both the superficial and deep layer of the cervical stroma at days 185 and 275 of pregnancy. Between day 275 of pregnancy and parturition, their numbers increased 166 and 17 fold in these layers respectively ($p<0.05$). At parturition, the neutrophils were mainly grouped in multiple poorly delineated foci. The number of eosinophils was nearly the same using Luna staining and Sirius red staining. Since these numbers remained low at all time points in both tissue layers, only the data obtained from the Luna staining are shown in Figure 6.1F.

Cytokine mRNA expression patterns

IL-1β mRNA expression did not differ between days 185 and 275 of pregnancy but increased 8 fold towards parturition ($n=7$, $p<0.05$, Fig 6.2A). IL-6 mRNA expression increased 6 fold between days 185 and 275 of pregnancy and stayed at that level at parturition. However, the differences between the time-points were not significant ($n=8$, Fig 6.2B). IL-8 mRNA expression was 5 fold (not significant) increased at day 275 of pregnancy and 430 fold increased at parturition, when compared to the level at day 185 of pregnancy ($n=10$, $p<0.001$, Fig 6.2C). IL-10 mRNA expression was 5 fold increased at day 275 of pregnancy ($p<0.001$) and 10 fold at parturition when compared to the level at day 185 of pregnancy, ($n= 8$, $p<0.001$, Fig 6.2D). TNFα mRNA expression decreased by 37 % at day 275 of pregnancy and 57 % at parturition when compared to the level at day 185 of pregnancy ($n=7$, $p<0.05$, Fig 6.2E).

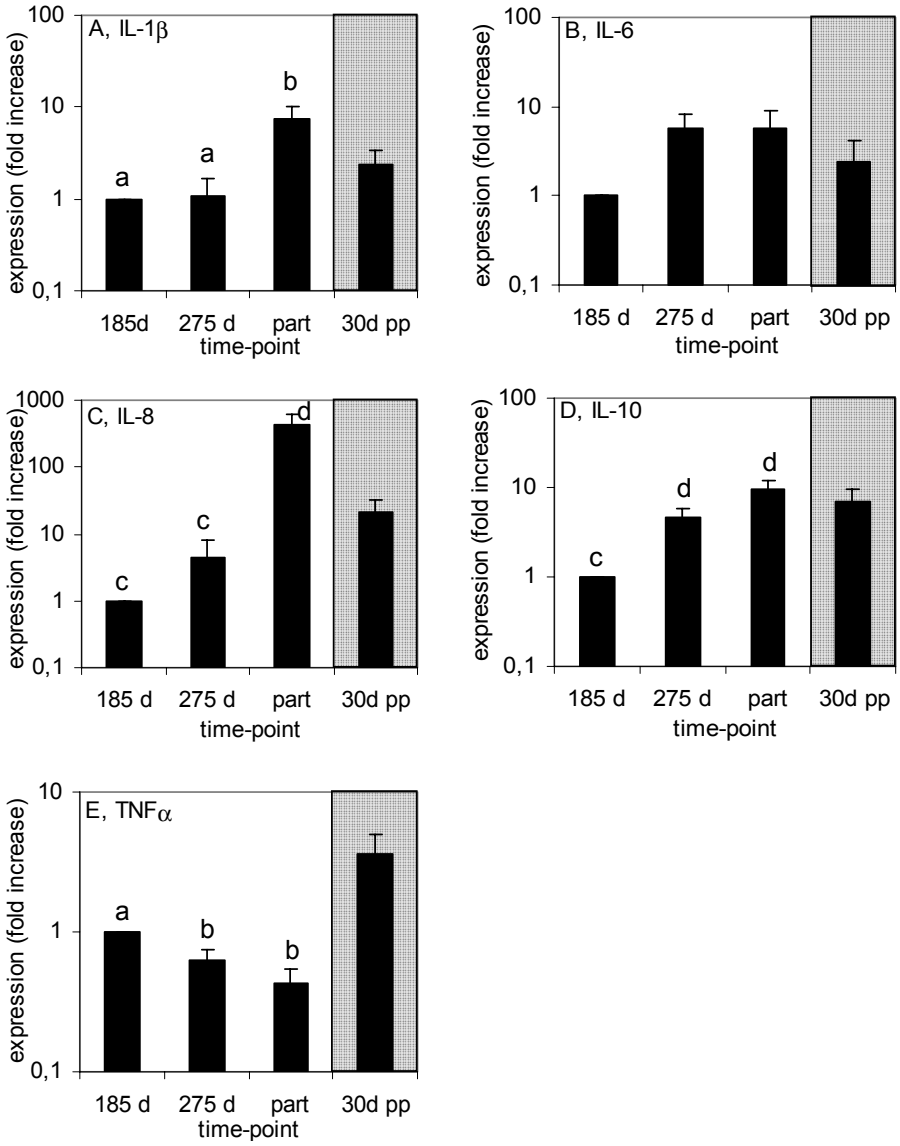

Figure 6.2 Mean (± SEM) mRNA expression of IL-1β (A, n=7), IL-6 (B, n=8), IL-8 (C, n=10) IL-10 (D, n=8) and TNFα (E, n=7) relative to the expression at day 185 of pregnancy in bovine cervical biopsies at several time-points. Biopsies were obtained at day 185 of pregnancy, day 275 of pregnancy, at parturition and 30 days after parturition. (a-b: $p < 0.05$; c-d: $p < 0.001$). Note the log scales of the y-axis and the different scales.

Discussion

In the present study, it was observed that large numbers of neutrophils had invaded the bovine cervix at parturition, while at that moment eosinophils remained scarce as determined by Luna staining, confirmed by Sirius Red staining. This is in accordance with studies in guinea pigs, mice, humans and sheep, which also reported the invasion of neutrophils at parturition [35-38]. In rats [39], however, and in cows, extended invasion of eosinophils in the parturient cervix was shown [10]. The discrepancy, between the numbers of eosinophils that were observed in the bovine cervix in the different studies is difficult to explain. It might have been caused by different methods or locations of sampling.

The more pronounced neutrophil invasion in the superficial part of the stromal layer as compared to the deep part agrees with a previous description in sheep [38] and, since in human cervices invading leucocytes were shown to secrete Matrix Metallo Proteinases (MMPs) which digest the collagen fibrils [11], this could also be the case in sheep and cows. However, in sheep, collagenolysis was not strictly limited to the sites of neutrophils influx [38]. This seems to indicate that not only neutrophils but also cells such as macrophages, cervical smooth muscle cells or fibroblasts secrete collagen degrading MMPs, potentially due to stimulation by IL-1 [40]. However, our observation that neutrophils were predominantly present in superficial foci immediately after expulsion of the calf could also indicate that neutrophil invasion was a consequence of epithelial damage during expulsion. On the other hand, superficial cervical neutrophil invasion has also been observed in parturient sheep before expulsion of the lamb [38], which points to a role of neutrophils in cervical ripening at parturition.

The mRNA expressions of cytokines were assessed using RT-PCR. Although pro-inflammatory cytokine production might have been regulated at different levels, we regard mRNA expression in this model to be a good measure for the actual cytokine production, since cytokines have a short half-life and transcriptional regulation is important [41,42]. At parturition, the IL-8 mRNA expression had increased 430 fold. Several types of cells may have produced this IL-8 initially. For instance macrophages that were resident in the cervical tissue or had invaded the cervix prior to the

neutrophils, as has been shown for mice, might have produced IL-8 [43]. IL-8 could also have been produced by invading neutrophils or resident mast cells that are numerous during pregnancy, as has been shown for guinea pigs [44]. Additionally, epithelial cells, fibroblasts or smooth muscle cells have been shown to secrete IL-8 [4,45]. The increased IL-8 mRNA expression at parturition was accompanied by an increased expression of IL-1β, which is in agreement with studies in other species [15,17,19]. However, in this study the IL-1β mRNA expression increased far less pronounced compared to IL-8 and the IL-6 expression did not even change significantly. In contrast to cytokine expression in inflammatory responses to infectious agents or their derivatives, in the present study, that of IL-1β and IL-6 seems to be of minor importance. This is possibly due to the activity of regulatory cytokines like IL-10, supposed to be active in constraining the negative consequences of the (pro) inflammatory cytokine cascade [46].

In contrast to what has been described regarding the involvement of pro-inflammatory mediators in cervical ripening [47-50], in the present study the mRNA expression of the pro inflammatory cytokine TNFα was significantly decreased at day 275 of pregnancy and at calving. This agrees with results of human studies, where cervical TNFα levels were not increased at parturition in contrast to IL-1, IL-6 and IL-8 [8,17,49,51,52]. A role for TNFα in cervical ripening has mainly been based on observations that experimental application of TNFα resulted in cervical ripening *in vivo* and the secretion of MMPs by cervical smooth muscle cells *in vitro* [40,48,53] or on the finding that high TNFα levels are present in amnion fluids during parturition, especially in cases of infections [16,54]. However, although this seems to imply that TNFα plays a role in cervical ripening, this might not be the case at physiological parturition.

In this experiment, relative to the level at day 185 of pregnancy, the mRNA expression of IL-10 in cervical biopsies had increased at 275 days of pregnancy and at parturition. This contrasts with the expression pattern in choriodecidual tissues where IL-10 was high during pregnancy and decreased at labor [21,22]. The high levels of IL-10 in the pregnant human uterus are thought to inhibit an inflammatory reaction and rejection of the

fetal allograft [55,56]. In contrast, the relatively low levels of IL-10, in combination with the relatively high level of TNFα that we found in the bovine cervix at day 185 of pregnancy, compared to day 275 of pregnancy and parturition might indicate that at this stage of pregnancy the immune system in the bovine cervix is activated, which is important in the defense against ascending infections [57]. We suggest that the higher levels of IL-10 at parturition may be crucial to limit the inflammatory reaction and prevent excessive tissue damage at final cervical ripening. This study assessed cytokine levels at 30 days after parturition. Future experiments with samples at different time-points after parturition would elucidate the involvement of cytokines during the course of postpartum cervical involution.

The present study showed that high numbers of neutrophils had invaded the bovine cervix at parturition, whereas at about 5 days before parturition these cells were virtually absent. Most likely, the prominent expression of IL-8 mRNA was responsible for attraction of these cells. It has not become clear which cells are responsible for the IL8 production and which events trigger the production. At the same time, the IL-1β expression was increased, but to a far lesser extent than IL-8, the IL-6 expression was even unchanged compared to earlier time-points and the TNFα mRNA expression had clearly decreased over the course of time. The latter events may be attributed to the activity of regulatory cytokines like IL-10 that, based on mRNA expression, seemed to have increased strongly in the last few days before parturition.

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7

Summarizing discussion

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7

Cervical ripening is a complex process of modification of cervical tissue to enable dilation of the cervix at parturition. It is evident that these two aspects of cervical function, ripening and dilatation, are not only driven by factors from outside the cervix, e.g. uterine contractions and mechanical stress due to growth of the fetus, but also by activity of cells within the cervix. To analyze the involvement of cervical smooth muscle cells, the studies of this thesis focused on the two most prominent layers of the cervix, the outer muscle layer, and the stromal layer, which contains smooth muscle cells in addition to fibrous tissue (Fig. 1.1). **Contraction** of the smooth muscle cells could either hamper or, conversely, promote cervical dilatation. Additionally, smooth muscle cells may play an active role in ripening by **secreting** cytokines or MMPs. The successive events that take place at cervical ripening during pregnancy and dilatation of the cervix at parturition are discussed in this chapter and summarized in Figure 7.1.

7.1 Cervical ripening

Cervical ripening was studied over a time course including late pregnancy, parturition and the post partum period using biopsies recovered from the caudal aspect of the bovine cervix at 185 and 275 days of pregnancy, at parturition and at 30 days after parturition. Besides a thin epithelial layer, the biopsies contained stromal tissue, that consisted of smooth muscle cells, fibroblasts and a considerable amount of collagen that has to be degraded before parturition [1]. These samples were analyzed for the presence and production of cytokines and MMPs, with specific attention to a possible role for smooth muscle cells in this process.

Cervical ripening and MMPs

Cervical ripening is divided into two stages; gradual ripening during the last months of pregnancy and final ripening which occurs as late as during parturition [2,3]. MMPs and their regulators, such as TIMPs, play a role in cervical ripening [4-6]. Stromal cells, fibroblasts and smooth muscle cells, all have the capacity to produce these MMPs and might be involved in collagen degradation during both gradual and final cervical ripening, whereas at final ripening the inflammatory cells that invade the cervix may form an additional source of MMPs.

In **chapter 5** the presence of MMPs in the bovine cervix, before and after leukocyte invasion was addressed, together with attempts to identify the cells responsible for their production. MMP-2 mRNA and protein expression were maximal at day 275 of pregnancy which was, on average, 5 days before parturition (Fig 7.1B). MMP-2 was present in both smooth muscle cells and the surrounding extracellular matrix (ECM). At parturition, MMP-2 protein expression was still high, and MMP-2 was secreted by the smooth muscle cells. Our data are in agreement with observations in women that cervical smooth muscle cells produce MMP-2 at term while MMP-9 was secreted by leukocytes [5]. The presence of MMP-2 in smooth muscle cells and ECM before parturition suggests that MMP-2 derived from smooth muscle cells contributes to degradation of collagen fibrils during the last period of gradual cervical ripening. This degradation of collagen could than cooperate with an increased presence of several non-collagenous molecules within the ECM such as hyaluronan, heparin sulphate and decorin, to decrease the strength of the collagenous tissue [7].

Gradual cervical ripening is a prerequisite for normal parturition [8] and it is likely that collagen degradation is an important part of this process. However, cervical collagen is not completely digested during gradual ripening, as has been shown by Breeveld et al [1]. We suggest that MMP-2 activity is precisely regulated at this stage to cause a moderate, but not complete digestion of collagen. Regulation of the activity of MMP-2 in the ECM may occur in several ways; 1) MMP-2 activation might be a rate-limiting step for collagen cleavage activity; 2) the capacity of MMP-2 to cleave collagen is low compared to other collagenases and 3) MMP-2 might be inhibited by binding of TIMPs [9,10]. Since TIMP-2 mRNA expression in the cervix was high at day 275 of pregnancy (**chapter 5**); we suggest that this TIMP in particular might regulate MMP-2 activity.

At parturition, granulocytes invaded the cervix and MMP-1 and -9 expression levels were increased (**chapter 5**, Fig 7.1B). The MMP-1 and MMP-9 might have been produced by the granulocytes themselves [5], or by other (stromal) cells after stimulation by the granulocytes. It is likely that a collaborative action of the different MMPs at parturition is essential to digest cervical collagen extensively and enable the cervix to dilate. Besides the

proteolytic effect, high levels of MMP-9 also inhibit smooth muscle cell contractility [11], which might contribute to relaxation of the stromal smooth muscle cells at parturition.

Cervical ripening and a secretory phenotype for stromal smooth muscle cells

Stromal smooth muscle cells produce MMP-2 during cervical ripening (**chapter 5**), and do not exhibit EMG activity at parturition (**chapter 2**). These two observations encouraged us to further characterize the stromal smooth muscle cells with respect to their contractile and secretory properties, as described in **chapter 4**. At parturition, the stromal smooth muscle cells were grouped together in bundles and expressed smooth muscle actin-α (SMA) and connexin-43, which indicate that the smooth muscle bundles are equipped to contract. However, neither connexin-43 and SMA mRNA expression nor protein expression, as shown by immuno-histochemical staining, had increased at parturition (Fig 7.1A). This indicates that the stromal smooth muscle bundles are not specifically primed to contract in a coordinated fashion during parturition. Furthermore, the decreased density of smooth muscle cells within the muscle bundles might negatively influence a contractile function of these bundles. This contrasts with the previously described hypertrophy and hyperplasia of the myometrium and a large increase in the number of gap junctions in the lead up to parturition [12], which enable it to exhibit strong and coordinated contractions. In the cervical stroma, abundant breakdown of the ECM by MMPs might further diminish the efficacy of contractility of the smooth muscle bundles, due to a lack of anchor points.

All smooth muscle cells in the cervical stroma expressed vimentin in addition to SMA, which contrasts with smooth muscle cells in other visceral organs [13]. Moreover, the proportion of co-expression of vimentin and SMA increased towards parturition. Since vimentin is involved in secretory processes [14] and is a characteristic of secretory smooth muscle cells [15], the data presented here suggest that stromal smooth muscle cells have a secretory function during cervical ripening, which is supported by the finding that these cells produce MMP-2. A change from a contractile into a secretory phenotype, known as phenotypic modulation, has been shown by

other investigators for vascular and vaginal smooth muscle cells [15-17] and for cervical fibroblasts [18] which share many features with smooth muscle cells. It is therefore likely that modulation to a secretory phenotype also occurs in cervical stromal smooth muscle cells during cervical ripening, whereas the same cells may have a contractile phenotype at other stages of reproductive activity.

Final cervical ripening and inflammation

The final ripening of the cervix has been described in various species as the result of an inflammatory process [19] in which cytokines play an important role [20]. The experiments described in **chapter 6** aimed to investigate the presence of inflammatory cells and several pro-inflammatory and anti-inflammatory cytokines in the bovine cervix. We observed that neutrophils but not eosinophils invaded the bovine cervix at parturition, as they do in other animal species and women [21-23]. In contrast, Wehrend *et al* (2004) described the invasion of eosinophils into the cervix of parturient cows [24]. There is currently no obvious explanation for the discrepancy between these two studies, but it might have been evoked by differences in the timing and the location or method of sampling. In the present study IL-8 expression increased strongly at parturition, and was probably responsible for attracting the neutrophils that directly or indirectly stimulated expression of MMP-1, MMP-9, IL-1β and further IL-8 at parturition [25], thereby playing a role in final cervical ripening [26]. This is in agreement with an immunohistochemical study on human cervical tissue [27] and with *in vivo* studies in guinea pigs which showed that intracervical application of IL-1 or IL-8 result in cervical ripening indistinguishable from physiological final ripening [28].

In contrast to IL-8 and IL-1, mRNA expression for IL-6 did not increase towards parturition, while the expression of TNFα was lower at day 275 of pregnancy and at parturition than at day 185 of pregnancy. This might be due to a regulatory effect of IL-10, which was present at a higher concentration on day 275 of pregnancy and at parturition than on day 185 of pregnancy. The patterns of IL-10 and TNFα expression observed during pregnancy and parturition differed markedly from what has been described for the uterus previously. In the uterus, IL-10 levels are high during

pregnancy and decrease before parturition while TNFα levels show an inverse pattern [29-31]. This illustrates a state of uterine immuno-tolerance during pregnancy, caused by progesterone, which stimulates IL-10 production by dendritic cells [32,33]. The cytokine expression pattern in the cervix indicates a more vigilant immune status during pregnancy. It might be that, in the cervix, the immuno-suppressive effect of progesterone was overruled by an immuno-stimulating effect of continuous exposure to the bacterial flora of the vagina. At parturition, IL-10 might attenuate the cervical inflammatory reaction and, thus, excessive tissue degradation [34]. The relatively decreased levels of TNFα that we found during calving in our study (**chapter 7**) indicate that the mechanism of physiological final ripening differs from that induced by bacterial infection or bacterial products, as addressed in previous experimental approaches, which strongly stimulate TNFα production [35-37]. At the onset of an inflammatory reaction the transcription of several different proteins, such as inducible nitric oxide synthase (iNOS, generating NO), Cyclooxygenase-2 (COX2, generating prostaglandins), pro-inflammatory cytokines and MMPs, increase in concert. An important common transcription factor for these genes is Nuclear Factor κ B (NFκB). NFκB therefore plays a central role in cervical ripening [38]. The activity of NFκB is inhibited when progesterone binds to the progesterone receptor, as is the case during pregnancy. Activation of NFκB in turn inhibits the activity of the progesterone receptor, and thereby decreases the inhibitory action of progesterone on cervical ripening [39,40]. TNFα and LPS are major NFκB stimulating agents [41] and have been shown to induce cervical ripening in cases of cervical or uterine infection. Since activation by LPS or TNFα is of limited importance during normal final cervical ripening (**chapter 6**) other, as yet unknown, factors must be involved in the activation of NFκB.

Progesterone is essential for maintenance of pregnancy in women and most domestic animals and, a decrease in the progesterone level or a decrease in the concentration of progesterone receptors leads to cervical ripening and the induction of parturition [42]. Application of progesterone receptor antagonists to pregnant women and cows induces cervical ripening and parturition while, on the other hand, supplementation of

progesterone in cows in which calving has been hormonally induced, prevents sufficient cervical ripening and dilatation [43]. It is likely that besides withdrawal of progesterone inhibition [44], a pro-inflammatory stimulus is required for the onset of an inflammatory reaction. Such a pro-inflammatory stimulus might be the presence of glucosaminoglycans, which have been shown to stimulate the production of MMPs and cytokines within the cervix [45], or the presence of activated macrophages of fetal origin that migrate to the uterus and express pro-inflammatory cytokines [46]. Since collagen degradation products have pro-inflammatory effects in synergy with IL-8 [47,48], our findings suggest that MMP-2 activity induces, or intensifies, cervical ripening by generating such collagen degradation products.

At bovine parturition, the cervix starts to dilate at a precisely defined time after uterine and cervical contractility have started to increase (**chapter 2**, [49]). This suggests that uterine or cervical contractions initiate cervical final ripening. However, It has been shown in parturient sheep and rats, *in vivo,* that ripening of the cervix, as judged by their biomechanical properties, occurs independent of uterine contractions [50-52]. Although the influence of uterine contractions on biochemical or inflammatory parameters of cervical ripening has not yet been assessed *in vivo*, it has already been shown, *in vitro,* that stretching of smooth muscle cells, such as occurs in cervical tissue under the influence of uterine contractions, stimulates the secretion of inflammatory mediators and MMPs [53-56]. Therefore, contractions of the uterus or cervix might substantially enhance final cervical ripening.

7.2 Cervical dilatation and smooth muscle cells

In **chapters 2 and 3** we quantified EMG activity patterns of the cervical outer muscle layer, cervical stromal layer and myometrium, together with the changes in cervical diameter before, during- and after calving. In this model, parturition was induced with PGF2α, which causes a decrease in maternal plasma progesterone levels that mimics the normal decrease in progesterone before spontaneous parturition, as discussed above.

Cervical dilatation

At 29 hours after PGF2α injection the caudal cervix started to dilate progressively, at a rate of 4 cm/h, until maximal dilatation was reached. This was comparable with previous observations, e.g. Dwarkasing *et al* [49], that showed that inducing parturition with PGF2α leads to calving under reproducible conditions. After expulsion of the calves, the diameter of the cervix changed in a biphasic way. At the start of the post-expulsion measurements, some 1.4 h after the birth of the calf, the diameter of the cervix measured about 6 cm. In the first 15 h after delivery, diameter increased and only after this initial increase did it finally start to decrease. The small diameter of the cervix immediately after fetal expulsion and its increase over the first 15 h contrasts with the results of a study by Wehrend *et al*. (2003) who described a large cervical diameter immediately after calving and a subsequent progressive decrease [57]. However, from 15 h after expulsion onwards, the cervical diameters in the two studies were almost exactly the same. The unexpectedly small cervical diameter that we initially measured after fetal expulsion might have been caused by collapse of the distended, flaccid cervical walls when the cow lies down. Alternatively, muscle activity from within the cervical stroma might have been responsible for this effect. The increase in cervical diameter during the first 15 h after parturition might also have been caused by a progressive increase in the rigidity of the cervical tissues to counteract the collapse of the cervical walls, or by the presence of parts of the retained placenta, propelled into the cervical lumen by the involuting uterus. On the other hand, the initial rather large cervical diameter reported by Wehrend *et al* (2003) might have been caused by ingress of air into the vagina during the introduction of measuring devices in standing cows. In our own study, placental retention might have influenced the cervical diameter mechanically. Placental retention might also have lead to a sustained secretion of inflammatory mediators, thereby influencing cervical consistency and involution. Ultrasonic assessment of cervical diameter after spontaneous calving without placental retention should indicate whether placental retention significantly affects cervical involution during the first two days postpartum.

Cervical EMG activity

Using bipolar stainless steel electrodes, EMG activity could be detected in the cervical outer muscle layer before, during and after parturition. The RMS values and duration of EMG activity of the cervical outer muscle layer began to increase simultaneously with that of myometrial EMG activity, at some 12 hours before the caudal cervix started to dilate (Fig 7.1D). During parturition, the pattern of cervical EMG activity changed gradually from a contracture to a contraction-like pattern, which was comparable to the changes found in the myometrium. After fetal expulsion, the EMG activity gradually declined in the cervical outer muscle layer as well as in the myometrium. These similarities between cervical outer muscle layer and myometrial EMG activity indicate that these two tissues function in unison.

Although similarities were observed between the EMG activity of the cervical outer muscle layer and the myometrium, there were also some differences: 1) Whereas the EMG activity of the myometrium increased steeply at parturition, and was well organized in bursts, the EMG activity of the cervical muscle layer increased more gradually and was less well organized in bursts. 2) The maximal EMG amplitudes within the cervical outer muscle layer did not increase at parturition while those in the myometrium did. 3) After fetal expulsion, the EMG activity in the cervical outer muscle layer decreased less steeply than those in the myometrium, and 4) The decrease in EMG activity in the cervical outer muscle layer was mainly due to a decrease in contraction frequency, while in the myometrium it was due primarily to a decrease in EMG amplitude. These differences point to a slightly different regulation of the muscular activity in these two tissues, in which gap junctions might play a role. In the myometrium, the number of gap junctions increases significantly just before parturition [12] allowing synchronization of the electrical activity of the smooth muscle cells [58]. It might be that such an increase in gap junctions does not occur in the cervical muscle layer. We observed that, at parturition, neutrophils and pro-inflammatory cytokines were present in the stromal layer of the cervix; this might have dampened muscle contractility, either by direct interaction with smooth muscle cells and/or by the stimulation of nitric oxide (NO) or production of inhibitory prostaglandins [59,60].

In future experiments, analysis of biopsies obtained from the myometrium and cervical outer muscle layer, recovered either by laparoscopy or a transvaginal route, should elucidate which factors regulate their activity at parturition.

EMG activity was absent in the cervical stromal layer before, during and after parturition (**chapter 2**), which markedly contrasts with our (unpublished) observations that this layer exhibits muscular contractions *in-vitro.* It is possible that the level of EMG activity was too small to be detected with the current electromyographic techniques. Additionally, the random orientation of the smooth muscle bundles within the stroma might have been the reason that their contractions were not associated with detectable EMG activity. Cytokines and MMP-9 present in the stromal layer at calving might have induced prostaglandin secretion, which might also have inhibited smooth muscle contractions [11,59,60]. Finally, the stromal layer appeared not to be specifically prepared for contraction at the time of parturition, as we observed in **chapter 4** and discussed above. One might suggest that the stromal smooth muscle cells contract in a non-coordinated fashion at parturition or, that they exert their contractile activity only at a later stage such as during the puerperium. Additionally, as witnessed by their vimentin expression and MMP-2 production, these cells appear to have an important function as secretory cells during gradual and final cervical ripening (**chapters 4 and 5**).

Cervical EMG activity and cervical dilatation

EMG activity of the cervical muscle layer was increased at parturition in cows, which is in agreement with a previous study in sheep [61,62] but does not confirm the suggestion by several authors that dilatation of the cervix at parturition is facilitated by a reduced contractility of the cervix [63-65]. Furthermore, it was observed in the current study that the cervical diameter could momentarily dilate, simultaneous with a burst of EMG activity in the cervical outer muscle layer. The correlation between these two events was evident after expulsion of the calf, and although not analyzed in detail, this correlation was also observed at parturition.

One might suggest that mechanical stretching, induced by cervical dilatation, evoked the EMG activity of the cervical outer muscle layer

[66,67]. In that case, dilatation of the cervix would mainly have been caused by postural changes of the calf or by uterine contractions that pushed the fetus or (retained) fetal membranes into the cervical lumen. However, a correlation between momentary cervical dilatations and cervical EMG bursts was observed immediately after fetal expulsion, when a large volume of fetal membranes is not usually present close to the cervix. Therefore, it is not likely that uterine pressure alone would have caused the cervical dilatations post partum and, although a direct influence of uterine contractions upon the cervical tissue cannot be excluded, it is likely that EMG activity of the cervical outer muscle layer is a cause, rather than a consequence of cervical dilatation.

If the contractions of the cervical outer muscle layer are the cause of the temporary dilations of the cervix, the question arises of how this occurs. One might postulate that the EMG activity of the longitudinal layer was overestimated as compared to that of the circular layer, because the former layer was closer to the EMG electrodes. However, it has been shown for the cervix of non-pregnant cows, *in vitro,* that the longitudinal muscle layer in fact has a higher maximal contraction force than the circular muscle layer [68]. In addition, in the dorsal side of the cervix of late pregnant cows, the longitudinal layer is twice as thick as the circular layer and the orientation of the muscular bundles within the longitudinal layer is more uniform than that within the circular layer [69]. Therefore, we hypothesize that, within the cervix, the longitudinal layer exhibits stronger contractions than the circular layer and has a dilating function during parturition. Cooperation between dilative activity of the cervical outer muscle layer and contractile activity of the uterus [70] seems imperative for expulsion of the conceptus and fetal membranes, and for cleaning of the uterus prior to the reestablishment of reproductive cyclicity.

7.3 Future experiments

The data presented in this thesis show that smooth muscle cells of the cervical stromal layer and outer muscle layer play different roles during pregnancy and at parturition. Stromal smooth muscle cells are involved in gradual and final cervical ripening and produce MMPs. Further studies of the regulation of MMP secretion in relation to the influx of inflammatory cells and cytokine production might lead to the development of tools to influence the process of cervical ripening. The outer muscle layer cooperates with the myometrium in cervical dilatation at parturition. This might have clinical implications since pharmaceuticals that affect uterine contractility might also affect contractility of the cervical muscle layer.

The experimental data in our studies were obtained both by analyzing serial cervical biopsies recovered during pregnancy and spontaneous calving, and by analyzing EMG signals and cervical dilatation in cows undergoing induced parturition. In future experiments, extension and combination of these methodologies could provide extra information about cervical function at pregnancy and parturition that could be of relevance for veterinary and human obstetrics. Since final ripening occurs at a very late stage, analysis of cervical biopsies obtained at several time points after the induction of parturition with PGF2α would provide more detailed insight into the sequence of events involved in leukocyte invasion, MMP and cytokine secretion. Causal relationships might be elucidated by an extension of this approach involving vaginal application of agents that might affect the ripening process, for instance by inhibiting or stimulating NFκB, COX2 or iNOS. After parturition, ultrasonic measurement of cervical diameter in cows without placental retention would help elucidate to what extent the presence of a retained placenta influenced the pattern of cervical involution reported in this thesis. Such puerperal studies should be combined with cervical biopsies to investigate the course of post partum involution of the cervix and uterus with respect to the expression and presence of MMPs, cytokines and leukocytes. Finally, since we (and others) have observed that the smooth muscle cells of the cervical stromal layer exert contractile activity *in vitro*, which could not be detected at calving *in vivo*, further *in vitro* analysis of the contractility of this tissue in organ bath studies before

and after the application of hormones and/or cytokines should help to clarify the regulation and possible function of this muscular tissue.

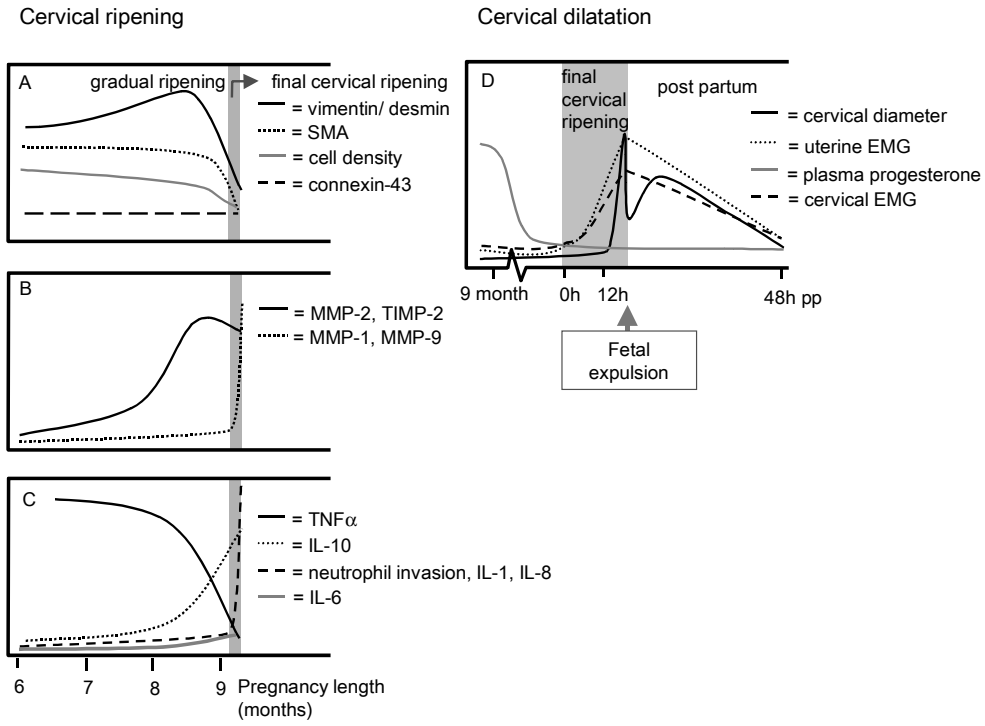

Figure 7.1. Graphs that summarize the pattern of events observed during cervical ripening and dilatation, based on the data presented in this thesis. A) Expression of markers that point to a contractile or secretory function of stromal smooth muscle cells (paragraph 7.1.2); B) Expression of MMPs and their regulators, involved in collagen degradation (paragraph 7.1.1); C) Expression of cytokines and the presence of neutrophils, pointing to an inflammatory reaction (paragraph 7.1.3); D) Cervical diameter around parturition, indicating a dilative effect of cervical and uterine contractile activity (paragraph 7.2). Graphs A-C are based on the analysis of biopsies of the caudal cervix, obtained at 185 and 275 days of pregnancy and at calving. Graph D is based on continuous recordings of the EMG activity and cervical diameter and multiple measurements of plasma progesterone concentrations.

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Abbreviations

AT	activity time of EMG activity
CDNA	copy-DNA
CML	cervical outer muscular layer
COXII	cyclooxygenase II
CSL	cervical stromal layer
ECM	extra cellular matrix
ELISA	enzyme linked immuno sorbent assay
EMG activity	electromyographic activity
GAPDH	glyceraldehyde 3-phosfate dehydrogenase
INOS	inducible nitric oxide synthase
IL	interleukin
LPS	lipopolysaccharide
MMP	matrix metallo proteinase
MYO	myometrium
NFκB	nuclear factor κB
NGS	normal goat serum
NLR	non-linear regression
NO	nitric oxide
OD	optical density
P	probability
PBS	phosfate buffered saline
PG	prostaglandin
PGF2α	prostaglandin F2alpha
RIA	radio immuno assay
RMS	root mean square value
RT-PCR	real time polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SMA	smooth muscle actin alpha
SMC	smooth muscle cell
TIMP	tissue inhibitor of matrix metallo proteinases
TNFα	tumor necrosis factor alpha

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Samenvatting in het Nederlands

A summary in Dutch

Zwangerschap (graviditeit) en baring (partus) zijn belangrijke processen in het leven van mens en dier. De baarmoedermond (cervix) speelt in deze processen een belangrijke rol. Tijdens de graviditeit moet de cervix goed gesloten zijn om te voorkomen dat de foetus uitgedreven wordt of dat bacteriën de baarmoeder (uterus) binnendringen. Tijdens de partus moet de cervix echter ontsloten zijn om de weg voor de foetus vrij te maken. Bij de mens zijn een slechte timing of verloop van de ontsluiting (dilatatie) belangrijke oorzaken van sterfte of morbiditeit in de postnatale periode en blijvende invaliditeit van het kind. Ook bij de koe is een goed verloop van de dilatatie belangrijk voor het afkalven.

De cervix bestaat voor een groot deel uit bindweefsel. Dit bindweefsel verkrijgt zijn stevigheid door het erin aanwezige collageen type 1. Dit moet voor en tijdens de partus zodanige veranderingen ondergaan dat het makkelijk rekbaar wordt. Dit proces wordt cervixrijping genoemd. Naast collageen en bindweefselcellen (fibroblasten) bevat de cervix ook gladde spiercellen. De hoeveelheid gladde spiercellen in de cervix varieert sterk tussen de verschillende diersoorten, maar bij het rund zijn het er relatief veel. In de rundercervix bevinden deze gladde spiercellen zich in twee verschillende gebieden: 1) in de buitenste spierlaag, die in feite een voortzetting is van de spierlaag van de uterus (myometrium) en 2) in de meer naar binnen gelegen bindweefsellaag (stroma), waarbinnen zij liggen gerangschikt in kleine spierbundeltjes.

Dit proefschrift beschrijft de rol die de gladde spiercellen in de cervix spelen bij de rijping en de dilatatie. Tijdens de dilatatie zouden ze een rol kunnen spelen vanwege hun contractiele eigenschappen. Daarnaast zouden ze een rol kunnen spelen in het rijpingsproces door het uitscheiden van stoffen die een ontstekingsreactie opwekken (cytokines), of enzymen die betrokken zijn bij de afbraak van bindweefsel (zogenaamde matrix metallo proteinases: MMP's).

De rijping van de cervix

De rol van gladde spiercellen bij de rijping van de cervix is onderzocht door bij koeien via de vagina biopten te nemen van het caudale (achterste) deel van de cervix. Gemiddeld duurt de draagtijd bij een koe 280 dagen. De biopten werden genomen op dag 185 en 275 van de dracht, zo snel mogelijk nadat het kalf was geboren, en op 30 dagen na het kalven.

Cervixrijping en MMP's

Het proces van cervixrijping kan worden onderverdeeld in twee fasen: 1) een geleidelijke fase, die plaatsvindt in de laatste maanden van de dracht, en 2) een eindfase, die plaatsvindt tijdens de partus en gekenmerkt wordt door een ontstekingsreactie. Van andere diersoorten is al bekend dat MMP's een rol spelen in de eindfase van de cervixrijping. Deze MMP's worden onder andere geproduceerd door witte bloedcellen (leucocyten) die op dat moment de cervix zijn binnengedrongen, maar ook gladde spiercellen zijn mogelijk een bron van MMP's. Omdat de gladde spiercellen altijd in de cervix aanwezig zijn, zouden ze MMP's kunnen afgeven op momenten dat de leucocyten nog niet aanwezig zijn, en dus al collageen af kunnen breken tijdens de fase van geleidelijke rijping, ruim voor de partus. In **hoofdstuk 5** hebben we de vraag onderzocht welke MMP's in de cervix aanwezig zijn op verschillende tijdstippen van de dracht en tijdens de partus. Het bleek dat MMP-2 mRNA en eiwitexpressie in de cervix al verhoogd waren op dag 275 van de dracht. Dit was gemiddeld vijf dagen voor het afkalven. Leucocyten waren de cervix op dat moment nog niet binnengedrongen. De MMP-2 was aanwezig in de gladde spiercellen en in het weefsel dat om de cellen heen ligt (extra cellulaire matrix, ECM). MMP-2 afkomstig uit gladde spiercellen kan dus mogelijk al collageen afbreken tijdens de geleidelijke fase van de cervixrijping. De afbraak van collageen door MMP-2 zou, samen met andere veranderingen in de samenstelling van de ECM, kunnen leiden tot verweking van de cervix, al ruim voor de partus.

De periode van geleidelijke rijping is nodig voor een goede ontsluiting van de cervix tijdens de partus, en het lijkt er dus op dat ook afbraak van collageen deel uitmaakt van dit proces. Toch is eerder al aangetoond dat het

collageen tijdens dit proces niet volledig afgebroken wordt. Bovendien zou een te sterke afbraak van het bindweefsel kunnen leiden tot vroegtijdige ontsluiting van de cervix. Daarom denken we dat de activiteit van MMP-2 tijdens deze fase nog enigszins wordt geremd, bijvoorbeeld door TIMP-2 (tissue inhibitors of MMP's, eiwitten die binden aan MMP en het daardoor inactiveren), waarvan de expressie hoog was op 275 dagen dracht.

Tijdens de partus zagen we dat er een verhoogde expressie was van MMP-1 en MMP-9. Deze MMP's spelen dus waarschijnlijk vooral een rol in de eindfase van de cervixrijping. De MMP's zijn mogelijk uitgescheiden door (binnengedrongen) leucocyten of door de reeds aanwezige cellen (fibroblasten en gladde spiercellen), in reactie op een stimulus vanuit de leucocyten. Het lijkt erop dat een ontstekingsreactie nodig is om de combinatie van MMP's te produceren die leidt tot een zodanige afbraak van collageen dat de cervix kan ontsluiten.

<u>Cervixrijping en een secretoire functie van de gladde spiercellen</u>

De gladde spiercellen in de binnenste bindweefsellaag (stroma) van de cervix produceren MMP-2. Bovendien hebben we aangetoond (hoofdstuk 2) dat het stroma geen electromyografische (EMG) activiteit vertoont tijdens en na de partus. Dit wierp de vraag op of de spiercellen in het stroma primair bedoeld zijn om te contraheren of juist om eiwitten te produceren. Daarom is in **hoofdstuk 4** een aantal eigenschappen van deze gladde spiercellen nader bestudeerd in de cervixbiopten die genomen zijn op de vier eerder genoemde tijdstippen.

Het bleek dat tijdens de partus de structuur van de spierbundeltjes in het stroma behouden bleef. Ook bleven de spiercellen smooth muscle actine alpha (SMA) en connexin-43 (een eiwit dat deel uit maakt van zogenaamde gap-junctions) tot expressie brengen. Dit duidt erop dat deze spiercellen in principe wel zouden kunnen contraheren tijdens de partus. De expressie van SMA en connexin-43 in het cervixstroma nam echter niet toe ten tijde van de partus, zoals dat wel gebeurt in het myometrium. Het spierweefsel in het stroma wordt kennelijk niet specifiek geschikt gemaakt voor contractie tijdens de partus. Daarnaast nam tijdens de partus de celdichtheid van de spierbundeltjes af en verloor het omringende weefsel waarschijnlijk zijn sterkte, zodat (eventuele) contractie van de spierbun-

deltjes de dilatatie van de cervix waarschijnlijk niet effectief meer zou hebben beïnvloed.

De spiercellen in het stroma bevatten vimentine, een intermediair filament dat karakteristiek is voor fibroblasten. De aanwezigheid van vimentine in de spiercellen kan erop duiden dat de belangrijkste functie van deze cellen een secretoire is en niet een contractiele. Een overgang van een contractiel naar een secretoir phenotype van gladde spiercellen is door andere onderzoekers reeds in bloedvaten en vaginawand vastgesteld. Dit zou ook in de cervix ten tijde van de partus het geval kunnen zijn.

<u>De eindfase van de cervixrijping en ontsteking</u>

De eindfase van de cervixrijping kan worden gekarakteriseerd als een ontstekingsreactie. Leucocyten dringen de cervix binnen, cytokines komen tot expressie, en het weefsel vertoont uiterlijk de karakteristieke tekenen van een acute ontsteking (tumor, rubor, calor, dolor, functio laesa). In het algemeen wordt verondersteld dat deze ontsteking nodig is om voldoende afbraak van het bindweefsel te verkrijgen, zodat volledige ontsluiting mogelijk is. Omdat bij het rund nog weinig bekend is over deze ontstekingsreactie, hebben we dit in **hoofdstuk 6** onderzocht.

Het bleek dat tijdens de partus veel neutrofiele granulocyten (een bepaald type leucocyten) het cervixweefsel waren binnengedrongen. Voor andere diersoorten was dit al eerder beschreven, en deze cellen bleken MMP's te produceren, die het collageen ter plaatse afbreken. Waarschijnlijk waren de neutrofiele granulocyten verantwoordelijk voor de toegenomen expressie van MMP-1 en MMP-9 die we in ons onderzoek tijdens de partus hebben aangetoond. Neutrofielen zijn vaak betrokken bij uitgesproken acute ontstekingsreacties en hebben een signaal nodig om het weefsel binnen te kunnen dringen. Interleukine-8 (IL-8) heeft een sterk aantrekkende werking op neutrofielen en de expressie van IL-8 was sterk verhoogd tijdens de partus. Bij de koeien tijdens de partus heeft IL-8 dus waarschijnlijk geleid tot de invasie van de neutrofielen, die zelf ook weer IL-8 produceren.

Tijdens de partus was de expressie van de ontstekingsmediatoren IL-1 en IL-6 slechts matig of helemaal niet verhoogd, en die van TNFα zelfs verlaagd. De oorzaak hiervan zou kunnen zijn gelegen in de verhoogde expressie van IL-10, want IL-10 is een regulerende cytokine dat de produc-

tie van pro-inflammatoire cytokines kan remmen. Als we de expressie van TNFα en IL-10 op dag 185 van de dracht vergelijken met die op dag 275 en tijdens de partus, dan valt op dat TNFα relatief hoog is op dag 185 en afneemt richting de partus, terwijl IL-10 relatief hoog is aan het einde van de dracht en tijdens de partus. Dit patroon is tegengesteld aan wat door andere onderzoekers werd beschreven voor de uterus. Men veronderstelt dat in de gravide uterus IL-10 de afweer remt, en op die manier voorkomt dat de foetus afgestoten wordt. De relatief hoge TNFα-, en lage IL-10-expressie in de rundercervix tijdens de dracht in vergelijking met de partus, zou erop kunnen duiden dat het immuunsysteem van de cervix geactiveerd is tijdens de graviditeit. Dit zou veroorzaakt kunnen worden door de voortdurende blootstelling aan bacteriën vanuit de vagina.

Progesteron is het belangrijkste hormoon voor het instand houden van de dracht, en daling van het plasmagehalte van progesteron is bij de koe het belangrijkste maternale endocriene signaal voor het opgang komen van de partus. Het wegvallen van de invloed van progesteron lijkt dan ook een belangrijke voorwaarde voor de eindfase van de cervixrijping. Echter, voor een ontstekingsreactie in het cervixweefsel moet ook een pro-inflammatoir signaal aanwezig zijn. Gladde spiercellen in de cervix zouden zo'n signaal kunnen afgeven, bijvoorbeeld door de secretie van glucosaminoglycanen. Maar ook de afbraakproducten van collageen kunnen een pro-inflammatoire werking hebben. Die producten zouden in de cervix aanwezig kunnen zijn door de activiteit van MMP-2, en dit zou aanleiding kunnen geven tot de ontstekingsreactie op het moment dat de invloed van progesteron afneemt.

Het is opvallend hoe goed de eindfase van de cervixrijping en de weeënactiviteit van de uterus in de tijd op elkaar zijn afgestemd. Bij de koe start de dilatatie van het caudale deel van de cervix 12 uur nadat de uterus verhoogde contractiliteit begon te vertonen, en voor de verschillende koeien verschilde dit maar weinig. Dit doet veronderstellen dat contracties van de uterus en/of de cervix een belangrijke rol spelen bij de uiteindelijke rijping van de cervix. Het is bij andere diersoorten *in vivo* aangetoond dat cervixrijping ook plaatsvindt als de cervix chirurgisch gescheiden is van de uterus, en dus niet rechtstreeks door de contracties van de uterus beïn-

vloed kan worden. Echter, hiermee is de invloed van contracties van de cervix zelf niet uitgesloten. Daarnaast is *in vitro* aangetoond dat oprekking van cervixweefsel leidt tot de afgifte van ontstekingsmediatoren en MMP's in het weefsel. Er zijn dus redenen genoeg om aan te nemen dat contracties van de uterus en/of de cervix rijping van cervixweefsel bij de partus stimuleren.

Cervixdilatatie en gladde spiercellen

In de **hoofdstukken 2 en 3** analyseerden we de EMG activiteitspatronen van de buitenste spierlaag en de stromalaag van de cervix, en we vergeleken die met de EMG activiteit van de uterus en met de veranderingen in de diameter van de cervix voor, tijdens en na het kalven. Hiervoor implanteerden we EMG elektroden op de uteruswand en in de cervix van de koe en brachten we echokristallen aan op het caudale einde van de cervix. De partus werd op dag 274 van de dracht geïnduceerd door een intramusculaire injectie met prostaglandineF2α. Dit veroorzaakt een sterke daling van progesteron in het bloed, vergelijkbaar met die voorafgaand aan een spontane partus. Deze partusinductie leidde er echter wel toe dat na de (normaal verlopende) geboorte van het kalf.bij alle koeien het foetale deel van de placenta vast bleef zitten

Dilatatie van de cervix

Het caudale einde van de cervix begon 29 uur na de prostaglandine injectie te ontsluiten met een snelheid van 4 cm/uur. Op het moment dat de maximale diameter was bereikt werden de echokristallen verwijderd en vond uitdrijving van het kalf plaats. Na de uitdrijving van het kalf werden de echokristallen opnieuw op de cervix aangebracht en werden de veranderingen in de diameter van de cervix gedurende de eerste twee dagen na de partus gemeten. De diameter was op het moment van aanbrengen reeds tot 6 cm afgenomen. Vervolgens nam deze tot 15 uur na de uitdrijving echter weer toe tot ongeveer 9 cm. Pas hierna trad een daling op, tot de cervix op 48 uur na de geboorte gemiddeld een diameter van 5 cm had bereikt. De relatief kleine diameter van de cervix direct na het kalven is mogelijk veroorzaakt door het tegen elkaar vallen van de tegenover elkaar liggende delen van de slappe cervixwand, op momenten dat de koe was gaan liggen waarmee lucht uit de geboorteweg werd geperst. Een

verhoogde spieractiviteit van de cervix, door irritatie tijdens het aanbrengen, zou ook geleid kunnen hebben tot dit effect. De geleidelijke toename van de diameter gedurende de eerste 15 uur zou verklaard kunnen worden door het feit dat de contraherende en kleiner wordende uterus de uterusinhoud (met vastzittende nageboorte) richting de cervix duwt en daarmee het lumen vergroot. Omdat de nageboorte gedurende de eerste 48 uur postpartum niet werd uitgedreven, zou de aanwezigheid van de placenta geleid kunnen hebben tot secretie van ontstekingsmediatoren die de eigenschappen van de cervix weer beïnvloed kunnen hebben.

EMG activiteit van de cervix

Met de roestvrijstalen EMG elektroden waren we in staat om duidelijke EMG signalen van de buitenste spierlaag van de cervix op te vangen. De EMG activiteit bleek tijdens de partus toe te nemen. Deze toename begon 12 uur voor de start van de dilatatie, vrijwel gelijktijdig met de EMG toename van het myometrium van de uterus. Er waren meer overeenkomsten tussen de EMG activiteit van de buitenste spierlaag van de cervix en die van het myometrium. Voorafgaand aan de inductie van de partus vertoonde zowel de cervix als het myometrium een EMG patroon dat bestond langdurige perioden met EMG activiteit van geringe amplitudo (zogenaamde contractures). Na de inductie veranderde dit patroon op beide locaties geleidelijk in een patroon met meer frequente en korte perioden van EMG activiteit. Direct na uitdrijving van het kalf vertoonden zowel myometrium als cervix nog sterke EMG activiteit, maar deze nam geleidelijk af. De overeenkomsten tussen de EMG patronen van de buitenste spierlaag van de cervix en de uterus, wijzen erop dat de twee weefsels in een zekere mate als een eenheid functioneren.

Er waren echter ook verschillen tussen de EMG patronen van de buitenste spierlaag van de cervix en de uteruswand. Ten eerste was de toename van de EMG activiteit in de uterus sterker dan die van de cervix. Daarnaast was het EMG patroon in de uterus veel regelmatiger dan in de cervix. Bovendien nam in de uterus de maximale amplitude van het EMG signaal toe, terwijl dat in de cervix niet het geval was. Ten slotte bleek de afname in EMG activiteit van het myometrium na de partus voornamelijk het gevolg van een afname in de amplitude van het EMG signaal, terwijl dit in de

cervix het gevolg was van een afname in de frequentie van de contracties. Deze verschillen duiden erop dat de regulatie van de activiteit van de buitenste spierlaag van de cervix verschilt van die van de uterus. Het is bekend dat gap-junctions (verbindingskanaaltjes tussen de cellen), opgebouwd uit connexin-43 eiwitten, een belangrijke rol spelen bij het voortgeleiden van contracties over de uterus. Het zou kunnen dat de expressie van connexin-43 in de buitenste laag van de cervix minder sterk toeneemt dan in de uteruswand. Een andere mogelijkheid is dat de activiteit van de spierlaag van de cervix wordt beperkt door de aanwezigheid van remmende prostaglandines of stikstof oxide (NO).

In de stromalaag van de cervix kon geen EMG activiteit gemeten worden. Dit is opmerkelijk, gezien het feit dat deze laag naast veel bindweefsel ook veel spiercellen bevat en dat deze laag, eenmaal uitgesneden en in een weefselbad gebracht, spontaan contracties vertoont. Het is mogelijk dat de contractiele activiteit van de stromalaag wordt geremd door de verhoogde aanwezigheid van ontstekingsmediatoren. Het is echter ook niet uitgesloten dat door de ongestructureerde en verspreide ligging van de spierbundeltjes met onze methodiek geen EMG signalen konden worden geregistreerd. Aan de andere kant hebben we elders in dit proefschrift ook aangetoond dat de gladde spiercellen in het stroma niet specifiek voorbereid zijn om te contraheren tijdens de partus, maar waarschijnlijk een secretoire functie hebben.

<u>EMG-activiteit van de cervix en dilatatie</u>

De spieractiviteit van de buitenste spierlaag van de cervix nam toe tijdens de partus. Dit past niet bij de gedachte dat het gladde spierweefsel van de cervix een rol speelt bij het gesloten houden van de cervix tot aan het einde van de graviditeit. Bovendien bleek na uitdrijving van het kalf dat perioden met EMG activiteit van de spierlaag van de cervix samenvielen met tijdelijke toenames van de diameter van de cervix. Nu zou men kunnen veronderstellen dat EMG activiteit van de cervix het gevolg en niet de oorzaak is van het dilateren van de cervix., Het is immers bekend dat glad spierweefsel bij mechanische stimulatie kan reageren met een contractie. Het is echter ook goed mogelijk dat de gemeten EMG activiteit van de cervix de oorzaak is van de cervixdilatatie want, gezien de positie van de

electroden, hebben we waarschijnlijk vooral de activiteit van de buitenste spierlaag gemeten, en deze laag heeft een longitudinaal verloop en daardoor een dilaterende functie. Dit effect hebben we nog niet in detail bestudeerd tijdens de dilatatie fase van partus. Maar het is goed mogelijk dat het ook dan aanwezig is. Het is goed voorstelbaar dat een dilaterend effect van de contracties van de gladde spiercellen van de cervix, samen met een drukverhogend effect van de myometriumcontracties, ertoe leidt dat de inhoud van de uterus efficiënt wordt uitgedreven.

Samenvattend: de spiercellen van de buitenste spierlaag van de runder-cervix van het rund zijn in functioneel opzicht een voortzetting van het myometrium. Ze hebben een contractiele functie en de toename van de EMG activiteit tijdens de partus is een sterke aanwijzing, dat ze en betrokken te zijn bij de cervixdilatatie. De gladde spiercellen in de stromalaag hebben een secretoire functie en spelen door secretie van MMPs waarschijnlijk een rol in de geleidelijke fase en de eindfase van de cervixrijping. Deze eindfase van de cervix rijping bij het rund vertoont kenmerken van een ontstekingsreactie zoals de instroom van neutrofiele granulocyten en de toegenomen expressie van ontstekingsmediatoren.

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Curriculum vitae

Erik van Engelen werd geboren op 16 juli 1970. Na het voltooien van zijn middelbare schoolopleiding ging hij in 1988 Biologie studeren aan de Landbouw Universiteit Wageningen. Hij studeerde af in 1993 met orientatie Organisme en een afstudeervak bij de afdeling Immunologie en Celbiologie. Na in 1991 als kanonnier zijn dienstplicht vervuld te hebben is hij in 1992 gestart met de studie Diergeneeskunde te Utrecht. Deze studie heeft hij in maart 1998 afgerond met als differentiatie Landbouwhuisdieren/ Dierlijke produktie. Vervolgens heeft hij fulltime in een landbouwhuis-dierenpraktijk gewerkt, totdat hij in het najaar 2000 aangesteld werd als junior-docent bij de afdeling Fysiologie van de Faculteit Diergeneeskunde. Hier heeft hij tot januari 2004 fulltime les gegeven waarna hij het, in dit proefschrift beschreven, onderzoek heeft gestart. Het onderzoek heeft plaats gevonden in samenwerking met de divisie Farm Animal Health, waar de experimenten met de koeien hebben plaats gevonden.

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