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Relationship Between Collagenase-Like Specific Activities in Placentome and the Level of Steroid Hormones in Retained and Non-Retained Fetal Membrane Cows

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Abstract: In this study Collagenase-Like Enzyme Specific (CLES) activity was measured in placentomes of cows with or without Retained Fetal Membranes (RFM) and its correlation with steroid hormones was also evaluated. Preparum dairy cows (n = 12) were randomly assigned to two groups. The control cows (n = 7) were calved spontaneously and released their fetal membranes within 12 h of calving and served as the Non-RFM group. Treatment cows (n = 5) received dexamethasone for induction of calving. All treated cows did not release their fetal membranes within 12 h after calving and served as the RFM group. In the Non-RFM group, blood samples were collected 2-3 days before calving and every 12 h thereafter until calving. In the RFM group, blood samples were collected immediately before dexamethasone injection and every 12 h thereafter until calving. In both groups two other samples were collected at around calving and 12 h after calving. Tissue samples were taken from placentomes of both groups, around 12 h after calving and CLES activity was determined. The results did not show any significant difference in CLES activity between the 2 groups. Serum estradiol-17β (E_2) concentration was not statistically different between the 2 groups but its changes over time between groups was found to be significantly different. A significant difference between the groups in Estradiol/Progesterone (E_2/P_4) ratios was also observed. The results of our study indicate that prepartum estrogens concentrations can influence collagenase activity in placentomes and may have an important role in maturation and release of fetal membranes.

Key words: Retained fetal membranes, collagenase, steroid, cow, CLES, placentomes

INTRODUCTION

Retention of the fetal membranes in the cow is normally defined as the condition in which the fetal membranes are not expelled within a period of 12 h after expulsion of the fetus (Chew *et al*., 1978; Takagi *et al*., 2002). Retained Fetal Membranes (RFM) are one of the most important complications during the postpartum period in cattle with economic consequences, because it affects herd health, milk production and reproductive efficiency (Eiler and Hopkins, 1992; Laven and Peters, 1996; Eiler *et al*., 1997; Takagi *et al*., 2002).

Towards the end of pregnancy, placental maturation is completed. In the cow, this process is characterized histologically by a number of changes that take place mainly in the maternal crypt epithelium. Placental maturation is supposed to be a need for the detachment and release of the fetal membrane (Boos *et al*., 2003).

Matrix Metalloproteinases (MMPs)-a family of zinc and calcium dependent proteloytic enzymes- and their activators and inhibitors play an important role during placental development, parturition, detachment in placentomes and release of fetal membranes, as they are responsible for the degradation of extracellular matrix components (Walter and Boos, 2001; Curry and Osteen, 2003). MMP-2 (72 kD gelatinase) and MMP-9 (92 kD gelatinase) can degrade collagen types IV and V, fibronectin, proteoglycans and gelatin (Woessner, 1991; Maj and Kankofer, 1997). Presence of MMP-2 and MMP-9 has been demonstrated in bovine placenta during parturition (Maj and Kankofer, 1997; Walter and Boos, 2001). It was postulated that collagenases play an essential role in release of fetal membranes (Walter and Boos, 2001; Curry and Osteen, 2003).

The etiology of RFM is not completely understood. It has been suggested that the main cause of the RFM is a defect in the breakdown of collagen fibers in the placentome (Eiler and Hopkins, 1992). This idea was supported by the successful treatment of RFM by intraumbilical injection of collagenase in cattle and horse (Eiler and Hopkins, 1992, 1993; Eiler *et al*., 1997; Haffner *et al*., 1998) and in human (Fecteau *et al*., 1998).

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It appears that collagenase and hyaluronidase together could contribute to collagen breakdown and separation of cotyledons and caruncles in postpartum cows (Eiler and Hopkins, 1992). Although the major emphasis is given to collagenases in the literature other proteases (gelatinases, stromelysins, membrane type enzymes) are also important during placental release (Eiler *et al*., 1997) and uterine involution (Eiler and Hopkins, 1993; Curry and Osteen, 2003).

The source of collagenase for expulsion of placenta is not known. It is possible that its source is the placentome or blood born substances (Eiler and Hopkins, 1992). It was suggested that preparturient changes in steroid hormones are responsible for MMP expression and action in the reproductive tract (Curry and Osteen, 2003; Shah *et al*., 2006). It is also possible that there is a relationship between changes in steroid hormones concentrations and collagenase activity in bovine placentomes during parturition.

Therefore, the present experiment was designed to determine 1) the collagenase-like enzyme specific activity in placentomes of cows with and without RFM and 2) its correlation with steroid hormones concentrations (E_2, P_4 and estriol).

MATERIALS AND METHODS

The animal groups: Twelve Holstein dairy cows that were used in this experiment were housed in teaching dairy farms of Shiraz University. They were 269 to 282 days pregnant. The cows were randomly assigned to the following two groups.

Control group (Non-RFM cows): Seven spontaneous calving cows that expelled their fetal membranes within 12 h after expulsion of the fetuses were selected as the control group (Chew *et al*., 1978; Takagi *et al*., 2002). Commencing ten days before the expected date of parturition, the clinical signs of parturition were checked regularly. Blood samples (10 mL) to assess serum steroid concentrations were taken from coccygeal vein 2-3 days before the calving and every 12 h thereafter until calving day.

Treatment group (RFM cows): Five prepartum cows received 20 mg dexamethasone (Aburaihan Pharmaceutical Co., Iran) intramuscularly for induction of calving and RFM. Blood samples were collected from coccygeal vein immediately before dexamethasone injection and every 12 h thereafter until calving, used to measure serum steroid concentrations.

In both groups, two other samples were taken at around calving and 12 h after parturition, at the time of placentomal tissue sampling.

Serum was separated after clotting at room temperature in both treatment and control groups. Serum samples were labeled for each cow and preserved at -20°C for subsequent assay of steroids.

Tissue sampling and processing: Tissue samples were taken from placentomes (cotyledons and caruncles) of RFM cows, whereas in Non-RFM cows only from maternal caruncles. Tissue samples were taken around 12 h after parturition (Al-Sadi *et al*., 1994) in both groups using an elongated effeminator via the vagina. Tissue samples were taken at this time to prevent disturbances in uterine contractions and release of placenta. At least 5-6 tissue samples were taken from gravid uteri of each cow in both groups. These samples were taken from different placentomes or caruncles.

In the RFM cows, placentomes were washed with cold saline solution and maternal and placental sites were separated by the method described by Kankofer *et al*. (1998). Fetal membranes of the Non-RFM cows were immediately transferred to the laboratory after expulsion. The tissue samples were taken from at least 5-6 large cotyledons of the gravid horns using scissor and forceps. Then these samples were washed with cold saline solution.

Minimum weight for maternal and placental tissue samples for measurement of collagenase activity was 0.5 g. To prevent decrease of enzymes activities, all tissue samples were first frozen at -20°C and stored at -70°C until assays (Busuttil *et al*., 1980; Kankofer *et al*., 1998).

Preparation of collagen suspension: Collagen suspensions were prepared according to the method described by Bleeg (1991). Collagen from bovine Achilles tendon (Sigma, C9879) was dissolved in 20 mM acetic acid. Reconstituted collagen fibrils were prepared by adding Hanks' Balanced Salt Solution (HBSS), 5 mM $CaCl_2$ and 3 mM sodium azide, pH 7.8, to an acid stock solution to produce a 1 mg mL^{-1} suspension. The solution was left overnight at 35°C which resulted in visible aggregates of collagen fibrils. To increase solubility, sonication was performed at 0°C for 5 min (30-sec intervals) with an MSE sonicator with amplitude of 15 μm. During sonication the temperature did not exceed 10°C.

Measurement of collagenase-like enzymes specific activity: All tissue handling was performed on ice. Normally, 1 g of tissue samples were used (0.5 g maternal

and 0.5 g fetal part). Because of the possibility of incomplete separation of fetal and maternal parts, equal part of both fragments was used in measurement of enzyme activity. All samples were finely minced manually, homogenized in 9 mL of HBSS, centrifuged at 3000×g for 15 min at 5°C and the supernatant was used as the source of enzyme.

CLES-activity was assayed by the method described by Busuttil *et al.* (1980). In this method the assay is based on the principle that collagenase and collagenase like enzymes are capable of releasing amino acids from native insoluble collagen when incubated for several hours at 37°C. One collagenase-like enzyme unit is defined as the amount of the enzyme that release amino acids equivalent to 1 μmol of L-leucine in the reaction mixture containing native collagen over an 18 h period at 37°C, pH = 7.8.

Two hundred microlitre of supernatant were added into a tube containing 2.8 mL collagen suspension (1 mg mL^{-1}). The reaction mixture was incubated at 37°C in a shaking water bath for 18 h. The reaction was terminated by ice bath immersing. The test and blank tubes were centrifuged at 3000×g for 20 min at 5°C. A blank sample was prepared in which tubes were immersed in ice without incubation at 37°C. An aliquot of 400 μL from resulting supernatant fluid was transferred to a test tube for the colorimetric determination of amino acids by the ninhydrin method according to Sun *et al.* (2006). The protein content of supernatants was determined using the Lowry method using bovine serum albumin as standard (Lowry *et al.*, 1951). The collagenase-like enzyme units were calculated by using the L-lucine standard curve for the ninhydrin colorimetric method. The CLES- activity was calculated by dividing of enzyme units by protein concentrations (units mg^{-1} protein).

In this study CLES-activity in the feto-maternal junction were compared between the RFM and control groups.

Steroid hormones assay: Serum concentrations of estradiol-17β (RIA Spectria Estradiol, Orion Diagnostica, Finland), progesterone (Spectria Progesterone RIA, Orion Diagnostica, Finland) and estriol (RIA Unconjugated Estriol, Immunotech, Czech Republic) were determined by radioimmunoassay method. Sensitivity and specificity of the estriol assay was 0.01 ng mL^{-1} and 100%, respectively. Sensitivity and specificity of the estradiol assay was 20 pmol L^{-1} and 100%, respectively. Sensitivity and specificity of the progesterone assay was 0.3 nmol L^{-1} and 100%, respectively.

Statistical analysis: Statistical analyses were done using SAS software, version 9.1. Results are presented as mean±SEM. Statistical analysis for CLES activity was carried out by the independent T-Test in the RFM and Non-RFM groups. The analyses of steroid hormone data were implemented using the PROC MIXED procedure and Tukey-Kramer test. The Pearson correlation test was used to assess the relationship between measurements of steroid hormones and CLES-activity. The $P<0.05$ was considered as statistically significant.

RESULTS

All treated cows (n = 5) calved about 27 to 49 h (mean = 40.8±9.12) after dexamethasone administration. They did not release their fetal membranes within 12 h postpartum and were considered as the RFM group. In the Non-RFM group (control cows) the fetal membranes were released within 12 h after parturition.

The mean CLES-activity in RFM and Non-RFM groups were 3.60±0.84 and 2.46±0.47, respectively ($p>0.05$) (Table 1).

The serum samples were arranged as five ranges of time in relation to the calving time (Table 2): 24-36 h prepartum, 12-21 h prepartum, 1-11 h prepartum, 1-9 h postpartum and 11-13 h postpartum. Concentrations of serum steroids (P_4, E_2 and esteriol) and their ratios (E_2/P_4 ratio and estriol /P_4 ratio) in RFM and Non-RFM groups that were compared on the same ranges of time are presented in Table 2 and Fig. 1 and 2.

There was no significant difference in the levels of serum P_4 between the two groups at the different time intervals ($p>0.05$). The serum P_4 concentrations of both the RFM and Non-RFM cows increased gradually ($p>0.05$) from 24-36 h prepartum (3.58±1.57 and 4.59±1.06 nmol L^{-1} respectively) to 12-21 h prepartum (6.40±2.30 and

Table 1: Biochemical parameters determined by method of collagenase activity if the RFM and non-RFM cows (Mean±SEM)

Cow	Group	Collagenase activity	Protein content (mg mL^{-1})	CLES activity (U mg^{-1} of protein)
1	Non-RFM cows	2.6	0.63	4.12
2		1.33	1.17	1.14
3		1.14	0.6	1.9
4		2.22	0.63	3.52
5		2.09	0.57	3.67
6		1.01	0.69	1.46
7		1.77	1.23	1.44
Mean (±SEM)		1.74±0.23	0.79±0.15	2.46±0.47
8	RFM cows	0.38	0.3	1.27
9		1.77	0.45	3.93
10		1.52	0.24	6.33
11		2.66	0.69	3.86
12		1.01	0.39	2.59
		1.47±0.38	0.41±0.08	3.60±0.84

Table 2: Mean serum concentration of steroids (mean±SEM) at different sampling times

Groups	Time [a]	Progesterone (P_4) (nmol L^{-1})	Estradiol (E_2) (pmol L^{-1})	Estriol (nmol L^{-1})	E_2/P_4 ratio	Estriol/P_4 ratio
Non-RFM (n)[b]	24 to 36 h	4.59±1.06 (4)	448.75±87.4 (4)	0.04±0.03 (4)	0.11±0.03 (4)	.005±0.004 (4)
RFM (n)	Prepartum	3.58±1.57 (5)	201.94±81.54 (5)	0.3±0.16(5)	0.06±0.003 (5)	0.50±0.39 (5)
Non-RFM (n)	12 to 21 h	5.75±1.27 (5)	525.10±121.64 (5)	0.03±0.02 (5)	0.09±0.007 (5)	0.003±0.002(5)
RFM (n)	Prepartum	6.40±2.30 (5)	289.76±95.07 (5)	0.08±0.04 (4)	0.06±0.02 (5)	0.02±0.005 (4)
Non-RFM (n)	1 to 11 h	3.52±1.05 (5)	451.2±66.05 (5)	0.04±0.02 (5)	0.16±0.03 (5)	0.01±0.003 (5)
RFM (n)	Prepartum	3.91±1.40 (4)	371.80±94.47 (4)	0.06±0.03 (4)	0.11±0.02 (4)	0.03±0.02 (4)
Non-RFM (n)	1 to 9 h	2.36±0.59 (7)	139.15±48.47 (7)	0.13±0.05 (7)	0.07±0.01 (7)	0.17±0.14 (7)
RFM (n)	Postpartum	2.13±0.47 (2)	76.85±20.15 (2)	0.01±0 (1)	0.04±0.002 (2)	0.01±0 (1)
Non-RFM (n)	11 to 13 h	0.49±0.36 (7)	22.50±7.36 (7)	0.11±0.03 (7)	0.15±0.05 (7)	1.53±0.65 (7)
RFM (n)	Postpartum	1.27±0.43 (5)	49.90±10.86 (5)	0.03±0.02 (5)	0.07±0.02 (5)	0.09±0.08 (5)

[a]: Time of parturition was considered to zero time, [b]: Number of serum samples are shown in parentheses. At different range of times, some samples were not collected

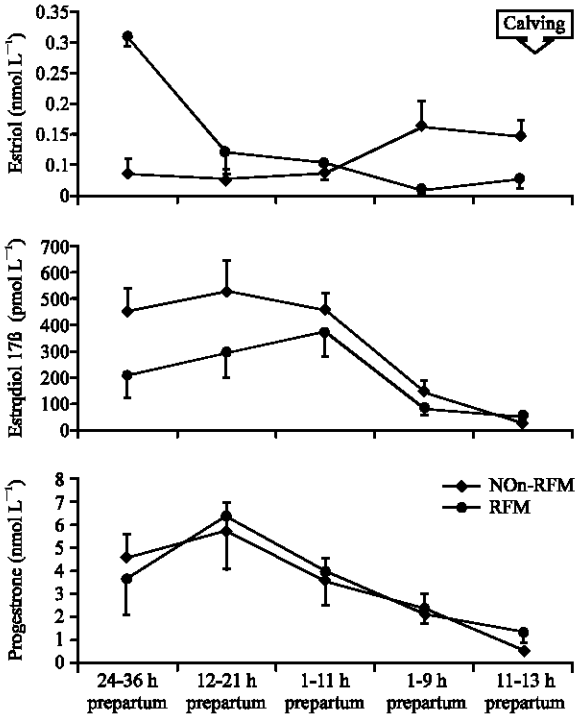

Fig. 1: Serum concentrations of steroids (P_4, E_2 and Estriol) in normal (Non-RFM) cows and in cows with Retained Fetal Membranes (RFM)

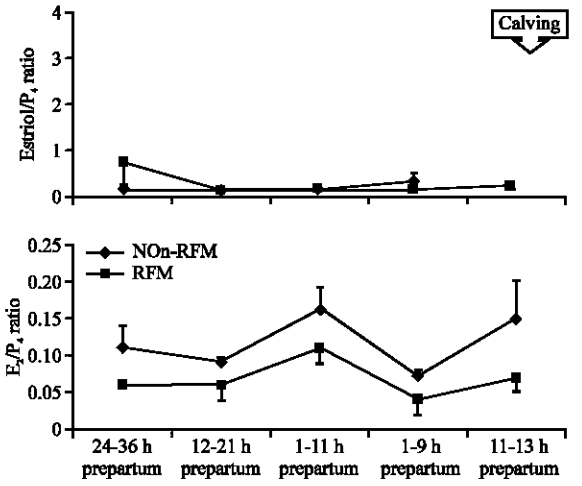

Fig. 2: Serum concentrations of steroids ratios (E_2/P_4 and Estriol/P_4) in normal (Non-RFM) cows and in cows with Retained Fetal Membranes (RFM)

5.75±1.27 nmol L^{-1}, respectively). Thereafter, they decreased gradually toward 11-13 h after calving (1.27±0.43 and 0.49±0.96 nmol L^{-1}, respectively). However, only mean serum P_4 concentration at 11-13 h after calving in the Non-RFM group was significantly ($p<0.05$) lower than the other sampling times (Fig. 1).

Serum E_2 concentrations were also not statistically different between the two groups ($p>0.05$) but it had a tendency towards a statistical difference at 11-13 h after calving ($p = 0.054$). Changes in serum E_2 concentration across groups and times were found to be significantly different ($p<0.05$). In the Non-RFM group, serum E_2 concentration increased gradually ($p>0.05$) from 24-36 h prepartum (448.75±87.4 pmol L^{-1}) to 12-21 h prepartum (525.10±121.64 pmol L^{-1}). Thereafter, they decreased significantly ($p<0.05$) to 11-13 h after calving (22.50±7.36 pmol L^{-1}). In the RFM cows, serum E_2 concentrations increased significantly ($p<0.05$) from 24-36 h prepartum (201.94±81.54 pmol L^{-1}) to 1-11 h prepartum (371.80±94.47 pmol L^{-1}). Thereafter, they decreased significantly ($p<0.05$) to 11-13 h after calving (Fig. 1).

The E_2/P_4 ratio was significantly different between the two groups and also its change over time between groups was significantly different ($p<0.05$). In the Non-RFM cows, the E_2/P_4 ratio was significantly different ($p<0.05$) between 1-11 h prepartum and 1-9 h postpartum. Also, in the RFM cows, this ratio at 24-36 h prepartum was significantly different ($p<0.05$) with 1-11 h prepartum. Change in estriol concentration and estriol/P_4 ratio was not significantly different between groups and over time between groups ($p>0.05$).

There was a weak correlation (r = 0.64, p = 0.065) between CLES-activity and P_4 concentrations in serum samples of both groups during 1-9 h postpartum. A negative significant correlation (r = -0.65, $p<0.05$) was found between CLES-activity and E_2 concentrations in serum samples of both groups during 12-21 h prepartum. There was a negative borderline correlation (r = -0.95, p = 0.054) between CLES-activity and serum estriol concentration during 12-21 h prepartum in RFM cows.

DISCUSSION

In comparison between RFM and Non-RFM cows, we found that the mean CLES-activity was slightly higher in RFM cows (3.60±1.88 versus 2.46±1.25) but this difference was not significant ($p>0.05$).

Our experiment confirmed the presence of collagenolytic activity in bovine placentomes, as found by Maj and Kankofer (1997) and Walter and Boos (2001). These enzymes may be utilized for the processes of releasing/retaining of fetal membranes (Maj and Kankofer, 1997) and uterine involution (Curry and Osteen, 2003).

The collagenase activity in RFM and Non-RFM cows determined was not statistically different in the present study. This result was similar to that reported by Walter and Boos (2001) who did not find any differences in MMP-2 and MMP-9 immunohystochemical distribution between RFM and Non-RFM cows. Although these researchers used prostaglandin-$F_{2\alpha}$ for induction of calving, we used dexamethasone. It was proposed that dexamethasone can interfere with collagenase activity in vitro (Sadowski and Steinmeyer, 2001). While we did not find any significant difference between CLES-activity in the two groups after dexamethasone injection, this might be due to low dexamethasone half-life in cattle (5.16 h) (Gaignage *et al*., 1991). It is also possible that stimulation of immune cells, which have a great amount of MMPs, causes increase in CLES activity of placentomes in the RFM group (Al-Sadi *et al*., 1994).

Our finding is in contrast with results published by Maj and Kankofer (1997). They found differences in enzyme activity of MMP-2 and MMP-9 between fetal and maternal sides of placentomes in RFM and Non-RFM cows; but these differences were not similar to all forms (proMMP and Active form MMP) of these enzymes and in both maternal and fetal sides (Maj and Kankofer, 1997).

It is likely that no difference in collagenase activity between RFM and Non-RFM cows, determined in our study, was related to infiltration of inflammatory cells (neutrophils and macrophages) in the retained placenta (Al-Sadi *et al*., 1994). These cells have high collagenase store (Woessner, 1991) and cause to increase collagenase activity in the uterus of RFM cows. Also, collagenase activity in Non-RFM cows may be decreased until the time of tissue sampling (12 h postpartum).

In this study serum P_4 levels were not significantly different between the 2 groups at the different time intervals. Chew *et al*. (1978) observed that serum P_4 concentrations decreased significantly following dexamethasone treatment. They found that the level of serum P_4 was significantly different between treated and non-treated cows that received dexamethasone. They also found that serum P_4 decreased faster and reached lower concentrations before calving in the RFM group compared with the untreated groups (Chew *et al*., 1978). Wischral *et al*. (2001) and Farzaneh *et al*. (2006) did not find any difference between serum P_4 concentrations in RFM and Non-RFM cows; although, the cows in their studies did not received any treatment before calving. However, there are many discrepancies regarding progesterone variation in RFM and Non-RFM cows (Laven and Peters, 1996).

In the present study, the serum E_2 concentrations were not different between the 2 groups at the different time intervals; although, it was found that their changes in the RFM and Non-RFM cows were significantly different. There were statistical differences among prepartum serum E_2 changes in RFM cows, whereas these differences were not found in Non-RFM cows. In both groups, serum E_2 concentrations were decreased significantly after parturition; although these concentrations in Non-RFM cows were marginally lower than RFM cows after parturition (at 11-13 h postpartum). Farzaneh *et al*. (2006) found that estrogen concentration was significantly lower in the RFM group, pre and postpartum. Lower estrogen levels at calving in RFM animals have been reported in some other studies (Kankofer *et al*., 1996; Wischral *et al*., 2001). The results of study by Chew *et al*. (1978) who found a significantly higher average E_2 concentrations in RFM cows following induction of calving by dexamethasone, is in contrast with our study. This might be due to the different timing of blood sampling or date of induction of parturition (Laven and Peters, 1996).

In spite of the fact that serum E_2 concentrations between RFM and Non-RFM cows were not significantly different, changes in their concentrations were significant in the different groups before parturition. Increases in serum E2 concentrations, until 1-11 h prepartum, were significant in RFM cows, whereas in Non-RFM cows were gradual. These results were similar to Shah *et al*. (2006) for certain reasons.

It seems that serum E_2 changes before 12-24 h prepartum are important factor in placental maturation, as Shah *et al.* (2006) suggested that estrogens, especially E_2, is responsible for stimulation and releasing of MMPs. Negative correlation between collagenase activity and prepartum serum E_2 concentrations in our study may confirm this suggestion.

In the present study E_2/P_4 ratios were significantly different between the groups; which is in some way comparable with the study carried out by Farzaneh *et al.* (2006). While, Wischral *et al.* (2001) did not find any significant differences in E_2/P_4 ratio in RFM and Non-RFM cows; although in their study, P_4 levels were similar between the two groups and E_2 levels were significantly lower in RFM cows. In the above studies, cows were spontaneously retained fetal membranes.

CONCLUSION

Results of the present study suggest that changes in concentrations of steroids (P_4, E_2) and their ratio were significantly different between RFM and Non-RFM cows during periparturient period. Also, there were correlation between steroid concentrations and collagenase activity in placentomes. It is proposed that in order to release of fetal membranes during physiological postpartum period, in addition to the necessity of increasing in collagenase activity to the adequate amounts, this increase need to be at the suitable time. Also, changes in steroid hormones may be related to collagenase activity with direct effects on placentomes or by an inflammatory process.

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