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ORIGINAL ARTICLE

Differential immunohistochemical expression of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in cow uteri with adenomyosis during follicular phase

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Abstract The present investigation was intended to show a different immunohistochemical profile of matrix metalloproteinase-2 and Tissue inhibitor metalloproteinase-2 in bovine uteri with adenomyosis during follicular phase. Uterine samples of 32 cows in reproductive age were taken from the medial third of one of the uterine horns and grouped according to the adenomyosis degree (superficial and deep). Tissue sections (4 μm) were incubated overnight at 4°C with monoclonal antibody for matrix metalloproteinase-2 and Tissue inhibitor metalloproteinase-2. Staining intensities were evaluated in the luminal epithelium, ectopic and dystopic endometrial tissue (stroma, capillaries and glands), endometrial-myometrial border, myometrium, myometrial vessels (middle tunic and endothelium). The matrix metalloproteinase-2 expression was higher for deep adenomyosis samples, showing a differential mean reactivity in superficial endometrium, myometrial vessels, myometrium adjacent to adenomyotic focus and endometrial-myometrial border ($P<0.05$). Moreover, matrix metalloproteinase-2 expression was higher in deep adenomyosis samples than that of Tissue inhibitor metalloproteinase-2 in almost all uterine structures analyzed (except for the endometrial and myometrial vessels and endometrial-myometrial border). The opposite was observed in the follicular phase, for both normal specimens and with superficial adenomyosis, where Tissue inhibitor metalloproteinase-2 expression was higher than that of matrix metalloproteinase-2. In conclusion, a differential pattern of matrix metalloproteinase-2 and Tissue inhibitor metalloproteinase-2 was observed in cow uteri with adenomyosis.

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Introduction

Uterine adenomyosis is a benign cell growth disorder characterized by dystopic location of endometrial tissue (stroma and glands) within the myometrium (Jubb and Kennedy 1987; McEntee 1990). Occurrences of reproductive disturbances in women (Devlieger and D'Hooghe 2003; Levgur et al. 2000) and animals (Matsuda et al. 2001; Mori et al. 2001; Ferreira et al. 2008; Moreira et al. 2008) have been associated to adenomyosis.

According to the literature, adenomyosis is occasionally found in cows as a congenital disorder (Jubb and Kennedy 1987) and also is found in bitches with cystic endometrial hyperplasia (McEntee 1990). The uterine adenomyosis was observed in heifers/cows culled at slaughterhouses by Monteiro et al. (2003), who observed the occurrence of 26.7%, by Moreira et al. (2006) founded 70%, and by Ferreira et al. (2008) where were observed 53% of adenomyosis in repeat breeder beef cows. These percentages are conflicting; however the adenomyosis is not an unusual finding in cows, as reported in the classical literature (Jubb and Kennedy 1987, McEntee 1990), and a scarcity of records of this dystrophy in cows may be justified to be a myometrial injury that does not make up endometrial biopsy samples.

Some studies have suggested that the primary cause of this disorder is the disruption of the endometrial-myometrial border, which consists of a specialized extracellular matrix (ECM) that separates these uterine layers. The tissue architecture is maintained by the combined action of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) (Curry and Osteen 2003).

MMPs are proteolytic enzymes that depend on Zn^{2+} and Ca^{2} to fully express their activities, and they are responsible for the degradation of ECM components, like collagens, laminin and fibronectin. MMP activity may be inhibited by TIMP. Both MMPs and TIMPs are controlled by ovarian steroids, cytokines and growth factors locally produced in the uterus. The imbalance of these enzymes has been implicated in pathological processes, like tumor invasion, angiogenesis and metastasis, and in the development of diseases characterized by excessive or deficient degradation of ECM, like aneurisms (Birkedal-Hansen et al. 1993; Bochsler and Slauson 2002; Curry and Osteen 2003) and adenomyosis (Mori et al. 2001; Matsuda et al. 2001).

Basal membrane of vessels, of endometrial glands and of luminal epithelium of bovine contains collagen type IV (Yamada et al. 2002). This membrane can be degraded by MMP-2 and MMP-9, and is larger in uterus of mice (Mori et al. 2001) and women (Inagaki et al. 2003) with adenonomyosis. The adenomyotic tissue invades the myometrium along the branches of blood vessels that emerge from the deep myometrium (vascular layer of myometrium). This invasion mechanism involves the direct degradation of the basal membrane of these vessels; however, the basal membrane of the endometrial parenchyma is maintained. This migration is made possible by uncoordinated protelolytic action of MMPs on MEC components (Mori et al. 2001; Matsuda et al. 2001).

This study aimed to show a different immunohistochemical expression of MMP-2 and TIMP-2 in bovine uteri with superficial and deep adenomyosis during the follicular phase.

Material and methods

Samples

The samples of the middle-third of the uterine horn were collected from 32 unpregnant cows, in reproductive age, from local slaughterhouses. Sample collection and preparation and the classification of the estrous cycle and degree of adenomyosis were carried out according to the method described by Moreira et al. (2007).

The phase of the cycle was estimated in the moment of cow's culled, with basis in the ovary morphology (presence or absent of ovarian follicules smallest than 8 mm) according Pavlock et al. (1992). The pieces were stored in refrigerated containers and sent to the laboratory. A sample of 5 cm long and 3 cm wide, was removal of the medial third of the uterine horns and opened with the serosal surface facing a paper filter, to prevent its folding, was fixed in buffered formalin 10% and after a minimum of 48 h, was processed by embedding in paraffin and submitted to hematoxylin-eosin and van-Gieson stain.

To help determine the intensity of endometrial tissue penetration through the myometrial fibers was utilized a method of classification recommended by Moreira et al. (2006), which followed the degree of infiltration of the endometrial glands into myometrium (adenomyotic focus) on: a)adenomyosis superficial—When the glandular infiltration did not go beyond the superficial myometrium, at a profundity corresponding to a diameter of three glandular units, and b) deep—when the implication glandular exceeded the superficial myometrium, at a profundity corresponding to the diameter of over three glandular units (Fig. 1a–c).

The samples were grouped as follows: Group I, formed by normal uterine sections (n=12), Group II, composed by uterine sections with superficial adenomyosis (n=13) and Group III, which comprised uterine sections with deep adenomyosis (n=7), all of them in the follicular phase.

Immunohistochemistry

Paraffin sections (4 μm in thickness) mounted on silane (Silano, Sigma-Aldrich, MO, USA) coated glass slides were rehydrated and endogenous peroxidase activity was blocked using 3 % H_2O_2 in distilled water for 30 min. Sections were then immersed in citrate (pH 6.0) buffer and boiled for 30 min at 98°C to retrieve masked antigens. After that, slides were maintained at room temperature for 20 min. Subsequently, slides were dried carefully using tissue paper, enveloped with Dako pen® (Dako, CA, USA) immersed in a blocking solution for 1 h [Trisma-NaCl (Vetec, RJ, Brazil) with 1% bovine serum albumin fatty acids free (BSA, Sigma-Aldrich, MO, USA)] and 1% skimmed milk powder (Molico™, Nestlé, SP, Brazil) for pre-incubation. Once removed from the blocking solution sections were incubated overnight at 4°C in humid chamber with monoclonal antibody anti-MMP2 (Calbiochem, Tokyo, Japan, clone Ab-4) and anti-TIMP-2 (Neomarkers, CA, USA, clone Ab-5) as a 1:100 dilution. On the next day, slides were washed (5 min) in TBS (Trisma-buffered-saline with Tween 20) and treated with the LSAB-HRP$^+$ kit (Dako, CA, USA) according to the manufacturer's instructions. After a final wash in TBS slides were developed in chromogen DAB kit (Dako, CA, USA) according to the manufacturers' instructions, counterstained with hematoxylin, dehydrated, hyalined and sealed with Permount® (Sigma-Aldrich, MO, USA). All the paraffin sections was duplicated and one of them was submitted like negative control (incubated with distillated water and with LSAB-HRP$^+$ and DAB kit) in the same assay.

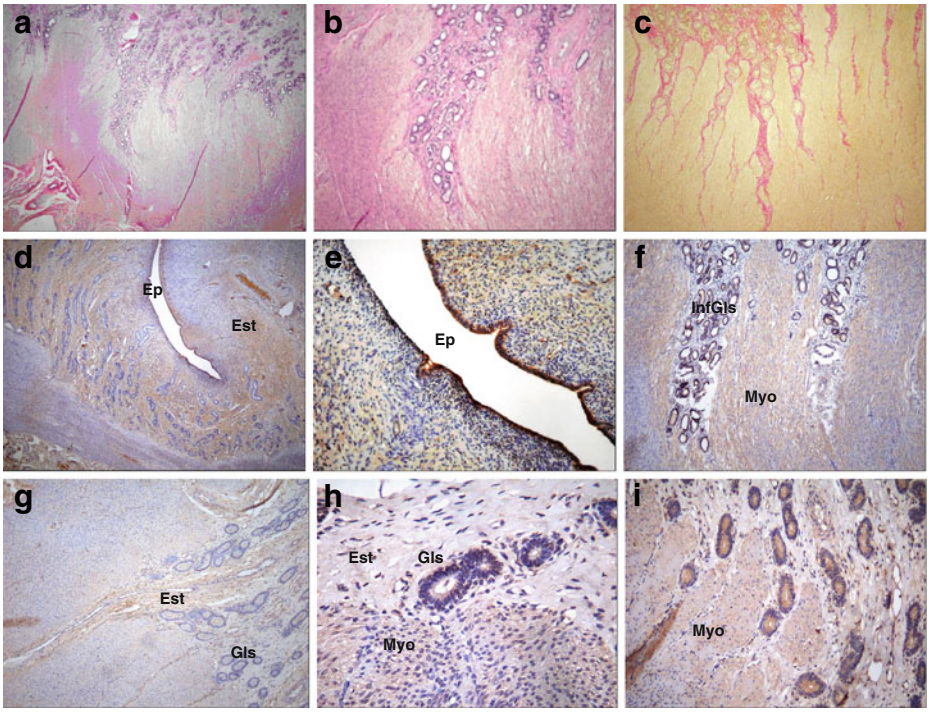

Fig. 1 Cow/uterine sections/Follicular phase; **a**/**b**—deep adenomyosis, endometrial tissue infiltrated into myometrium, 100× and 400× magnification, respectively, hematoxylin and eosin; **c**—deep adenomyosis, special van-gieson's stain, muscle fibers stained in yellow and collagen stained in pink, 200× magnification; **d**–**i**) immunohistochemistry-DAB/ counterstaining Hematoxylin. D—Normal uterus section, immunolabeling for MMP-2 in endometrial stroma (++) and surface epithelium (+++) and absence in endometrial glands, endometrial-myometrial border and myometrium, 400× magnification; **e**—Superficial adenomyosis, immunolabeling for MMP-2 in superficial epithelium (+++) and endometrial stroma (+), 200× magnification; **f**—Deep adenomyosis, immunolabeling for MMP-2 in deep and infiltrated endometrial glands (+++), myometrium adjacent to the adenomyotic focus (++), 100× magnification; **g**—superficial adenomyosis, immunolabeling for TIMP-2 in deep glands (++)—note apical reactivity—deep and infiltrated stroma (++), 100× magnification. **h**- Superficial adenomyosis, immunolabeling for TIMP-2 in endometrial glands (+) and deep stroma (+) and muscle fibers of the endometrial-myometrial border (++), 400× magnification; **i**- Deep adenomyosis, immunolabeling for TIMP-2 in deep endometrial glands and glands infiltrated in the myometrium (+++), endometrial vessel (++) and myometrial fibers adjacent to the adenomyotic focus (++), 200× magnification—legend: Ep—Superficial epithelium; Est—Endometrial stroma; Vs—Myometrial Vessels; Gls—Endometrial glands; Myo—Myometrium; InfGls—Endometrial glands infiltrated in the myometrium; Bord—Endometrial-myometrial border

Data analysis

The staining intensities were examined by light microscopy (Olympus BX41, MI, USA) in luminal epithelium, endometrial stroma (superficial and deep), endometrial capillary, endometrial glands (superficial and deep), endometrial tissue within myometrium (gland and stroma), endometrial-myometrial border, myometrium sheaves and myometrium vessels (middle tunic and endothelium), in three randomly chosen microscopic fields (200×), and were scored from negative (0); weak (+); moderate (++) to strong (+++).

The intensity means of each structure analyzed were compared using the Kruskall-Wallis test and the Mann-Whitney test. Values under 0.05 were considered statistically significant.

Results

Immunoreactivity pattern

All uterine sections presented reactivity for MMP-2 and TIMP-2. Immunoreactivity varied from weak to strong across the tissue structures of one same slide. The immunoreactivity pattern was cytoplasmatic, finely granular and several times projected apical projections of luminal and glandular epithelial cells (Fig. 1).

Follicular phase

The uterine sections with deep adenomyosis showed the highest mean intensity of reactivity for MMP-2 in almost all tissue structures analyzed, followed by those with superficial adenomyosis and normal sections (Table 1). In endometrial vessels and endometrial stroma infiltrated in myometrium, a higher reactivity mean for MMP-2 was observed in sections with superficial adenomyosis (Table 1–Fig. 1e). The superficial epithelium (peak expression), myometrial vessels, myometrium adjacent to adenomyotic focus and endometrial-myometrial border showed differences between means ($P<0.05$), showing high intensity of immunoreactivity for MMP-2 in sections with deep adenomyosis (Table 1–Fig. 1f).

The highest mean intensity of immunoreactivity for TIMP-2 in uteri with superficial adenomyosis was seen in the superficial epithelium, superficial stroma of the endometrium, deep glands and infiltrated into myometrium (Table 1). In turn, in uteri with deep adenomyosis, the endometrium and myometrium vessels exhibited highest means, but low intensity of reactivity in all uterine sections ($P>0.05$) (Table 1). The normal uterus exhibited high expression of TIMP-2 in superficial endometrial glands, endometrial deep stroma and myometrium; however, samples did not exhibit differences in mean immunoreactivity of all structures analyzed (Fig. 1g-i).

The normal uteri and uteri with superficial adenomyosis showed a tendency to exhibit higher mean intensity of immunoreactivity for TIMP-2 than for MMP-2, except in endometrial vessels and endometrial stroma infiltration in the myometrium (in sections with superficial adenomyosis), although the intensity of reactivity was low for both enzymes. Different mean MMP-2 and TIMP-2 expression values were observed for the superficial epithelium, endometrial glands and endometrial-myometrial border between normal uterine sections ($P<0.05$) and endometrial glands that were superficial, deep and infiltrated in the myometrium ($P<0.05$) of the uteri with superficial adenomyosis (Table 1).

The opposite was observed in uteri with deep adenomyosis, where higher mean immunoreactivity intensity was observed for MMP-2 than for TIMP-2 ($P<0.05$) (Table 1), with the exception of endometrial vessels and myometrium, and endometrial-myometrial border.

Discussion

Uterine adenomyosis is a benign lesion that occurs spontaneously in humans and animals, though it is considered a severe disorder in women, due to the intense hemorrhages, pain, colic and sterility (Levgur et al. 2000; Parrot et al. 2001). In bovines, the adenomyosis has been less frequently reported (Moreira et al. 2007).

Table 1 Immunohistochemical distribution of reactivity intensity of immunolabeling for MMP-2 and TIMP-2 normal bovine uteri and in bovine uteri with superficial and deep adenomyosis in the follicular phase of estrous cycle

Tissue structures	Normal uterine sections (n=12)		Uterine sections with adenomyosis			
			Superficial (n=13)		Deep (n=7)	
	MMP-2	TIMP-2	MMP-2	TIMP-2	MMP-2	TIMP-2
Superficialepithelium	*+++(4)/++(2)/+(6)a	+++(8)/++(4)	*+++(5)/++(4)/+(2)/0(2)a	+++(10)/++(3)	*+++(7) **b**	+++(4)/++(2)/+(1)
Endometrial superficial stroma	+++(2)/++(2)/+(1)/0(7)	+++(2)/++(1)/+(4)/0(5)	+++(5)/++(2)/+(2)/0(4)	+++(6)/++(2)/+(3)/0(2)	+++(1)/++(4)/+(1)/0(1)	+++(2)/++(2)/+(2)/0(1)
Superficial endometrial glands	++(3)/+(6)/0(3)	+++(4)/++(7)/+(1)	+++(1)/++(4)/+(5)/0(3)	+++(5)/++(6)/+(1)/(0)	+++(2)/++(3)/+(1)/0(1)	+++(1)/++(4)/+(1)/(0)
Deep endometrial glands	++(3)/+(2)/0(7)	+++(1)/++(1)/+(1)/0(9)	+++(2)/++(3)/+(4)/0(4)	+++(5)/++(5)/+(3)	+++(1)/++(3)/+(2)/0(1)	+++(1)/++(1)/+(4)/0(1)
Endoemtrial vessels	+++(1)/++(5)/0(6)	+++(2)/++(6)/+(1)/0(3)	+++(2)/++(2)/0(9)	+++(1)/++(4)/+(2)/0(6)	+++(2)/++(3)/+(1)/0(1)	++(3)/+(2)/0(2)
Deep endometrial stroma	+++(2)/+(5)/0(5)	+++(1)/++(1)/+(3)/0(7)	+++(2)/++(3)/+(3)/0(5)	+++(2)/++(1)/+(5)/0(5)	+++(1)/++(1)/+(1)/0(4)	+++(2)/++(1)/0(4)
Myometrium	+++(1)/++(2)/+(1)/0(8)	+++(3)/++(5)/0(4)	++(2)/+(3)/0(8)	+++(1)/++(4)/+(2)/0(6)	+++(3)/+(2)/0(2)	++(1)/+(1)/0(5)
Myometrium vessels	*+++(1)/++(2)/+(1)/0(8) a	++(4)/+(3)/0(5)	*+++(1)/++(3)/+(3)/0(6) a	+++(4)/++(2)/+(3)/0(4)	*+++(2)/++(2)/+(3) b	+++(2)/++(3)/+(2)
Endometrial glands infiltrated into myometrium			++(2)/+(6)/0(5)	+++(1)/++(6)/+(6)	+++(2)/++(4)/+(1)	++(1)/+(4)/0(2)
Endometrial stoma infiltrated into myometrium			+++(1)/++(4)/+(3)/0(5)	+++(1)/++(1)/0(11)	++(1)/+(4)/0(2)	+(1)/0(6)
Myometrium adjacent to the adenomiotic focus			++(1)/+(4)/0(8)a	++(3)/+(7)/0(3)	+++(2)/++(4)/+(1)b	++(1)/+(3)/0(3)
Endometrial-miometrial border	*0(12) a	++(8)/+(4)	*++(4)/+(2)/0(7) b	+++(2)/++(3)/+(5)/0(3)	*+++(1)/++(2)/+(2)/0(2) c	++(4)/+(2)/0(1)

Score for the intensities of immunolabeling: 0 negative; + weak positive; ++ moderate positive; +++ strong positive; MMP-2—Matrix Metalloproteinase-2 and TIMP-2—Tissue Inhibitor Metalloproteinase 2. *P<0,05 by Kruskall-wallis test. Letters in lines represent significantly different values in the Mann-Whitney test (p<0.05)

The mechanisms of adenomyosis progression have not been fully elucidated, though studies have indicated that this dystrophy is caused mostly by degradation and abnormal reconstitution of uterine elements adjacent to the endometrial-myometrial border due to the differences in MMPs and TIMPs activities, which allows endometrial tissue to invade the myometrium (Mori et al. 2001; Matsuda et al. 2001; Hirata et al. 2002; Yang et al. 2009).

In the follicular phase MMP-2 expression reached the highest values in deep adenomyosis samples, in comparison to normal tissue sections and superficial adenomyosis samples, with high reactivity observed in superficial epithelium, myometrial vessels, myometrium adjacent to the adenomyotic focus and endometrial border ($P<0.05$). Apart from this, the MMP-2 expression was higher than TIMP-2 expression in almost all uterine structures with deep adenomyosis (except in endometrial and myometrial vessels and endometrial border) analyzed in the present study. The opposite was observed in normal uterine sections and in those with superficial adenomyosis, for which TIMP-2 expression was higher than MMP-2 expression in the follicular phase. It is possible to hypothesize that this higher TIMP-2 expression may have acted as an inhibitor of the infiltration of endometrial tissue, playing a role in the maintenance of the normality of the uterine structures.

These data indicate that MMP-2 expression is increased in uteri presenting deep adenomyosis in the follicular phase, and that MMP-2 effects are more intense than the inhibitory effects of TIMP-2, since TIMP-2 expression was lower, which indicates a higher proteolytic MMP-2 activity.

MMP-2 activation takes place as a two-step process. In the first step the latent precursor of MMP-2 is cleaved by MT1-MMP and produces an intermediary form of MMP-2. MT1-MMP is a transmembrane protein, and the increase in MT1-MMP expression activates MMP2 on the cell surface, a process required for cell invasion, while TIMP-2 plays a dual role in the control of MMP-2 activation. A TIMP-2 and MT1-MMP complex is required for the activation of MMP-2. Yet, TIMP-2 alone form stable complexes with MMP-2 active site, and acts as a specific MMP-2 inhibitor (Chung et al. 2002; Curry and Osteen 2003). Therefore, MMP-2 activation depends on a fine balance between pro-MMP-2, TIMP-2 and MT1-MMP, that is, pro-MMP-2 does not bind to MT1-MMP in the absence of TIMP-2. Thus, if it is not activated, it remains in a latent state. In turn, given an excess of TIMP-2 the direct inhibition of pro-MMP2 takes place, and it does not bind to MT1-MMP (Curry and Osteen 2003). This explains occasional differences between TIMP-2 and MMP-2 expressions observed in the present study.

The fact that MMP-2 is activated in the cell membrane by MT1-MMP and TIMP-2 explains the cytoplasmatic and apical immunoreactivity in some cases, for both enzymes.

The degradation of ECM by MMPs is counterbalanced by TIMPs, which form a 1:1 complex with MMPs. Therefore, the equilibrium in the MMP:TIMP ratio may be an important regulator in the remodeling process of uterine MEC. Increased TIMP-2 levels indicate the inhibition of MMP-2 activity, while the lower TIMP-2 expression—as compared to MMP-2 expression—indicates the activation of pro-MMP-2 (Curry and Osteen 2003).

Previous studies (Moreira et al. 2007) have observed that during the luteal phase the incidence of adenomyosis as well as the degree of infiltration of the adenomyotic focus were more substantially increased, which suggests the influence of progesterone in the depth the focus may reach. This suggests an intensification of adenomyosis during the luteal phase, as well as a possible regression during the follicular phase.

The occurrence of adenomyosis is secondary to a previous dysfunction in the eutopic endometrium, which in turn translates as the capacity to resist to respond to the

progesterone signaling pathways (Lessey et al. 2006) and to respond too intensely to estradiol pathways. This leads to the inability to regulate the proteolytic activity of matrix metalloproteinases and to the consequently more expressive increase in cytokines, growth factors and activation of the matrix of metalloproteinases. These changes lead to the loss of uterine homeostasis, which affords the migration of endometrial tissue (Osteen et al. 2005).

The immunoreactivity pattern observed in the present study was similar to that reported by Walter and Boos (2001), except for the expression of MMP-2 in the luminal epithelium, particularly in specimens with deep adenomyosis (a peak in all samples, $P<0.05$).

Although no significant difference was observed, the deep endometrial stroma showed high reactivity in uterine sections in the initial and middle luteal phases, side by side with a higher MMP-2 expression in superficial adenomyosis. These data allow supposing that there is an increase in MMP-2 activity in this uterine region during the inception of adenomyosis, which would lead to the destruction of the endometrial-miometrial border and to the resulting migration of endometrial tissue across endometrial fibers.

The high MMP-2 expression in mesenchymal structures (myometrial and endometrial vessels, myometrial fibers, endometrial border and endometrial stroma infiltrated in the myometrium) observed in the present study corroborate the findings by Mori et al. (2001) and Matsuda et al. (2001). In those studies, the migration process was reported to occur along the branches of blood vessels that emerge in the myometrium, possibly mediated by the collagen breakdown promoted by MMP-2, which has increased expression in uteri of cows with adenomyosis.

The data obtained in the present study suggest that the expression of these enzymes in uteri of cows with adenomyosis is different from that observed in normal uteri, and that they are significantly similar to the expression of the enzymes in women (Inagaki et al. 2003; Yang et al. 2009) and female mice (Mori et al. 2001; Matsuda et al. 2001). Apart from this, MMPs and TIMPs are directly involved in the endometrial receptivity and embryo implantation in bovines (Hirata et al. 2002; Walter and Boos 2001; Hashizume et al. 2003; Hirata et al. 2003) and the imbalance in these enzymes' expressions may be associated to adenomyosis in cows, even leading to reproductive failure in the species.

These results indicate that the MMP-2 expression is higher in uteri of cows affected by deep adenomyosis and the TIMP-2 expression is higher in uterine sections with superficial adenomyosis, in the follicular phase.

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