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MATRIX METALLOPROTEINASES (MMPS) EFFECT ON ENDOMETRIUM IN CYCLIC COWS

A. N. M. I. Rahman[1], L. Y. Asad[2], B. F. Amy[3] and M. M. Hossain[4]

ABSTRACT

The aim of this study was to investigate the probable hormonal influence on the expression of matrix metalloproteinases (MMPs) in cow endometrial tissue. The cow uterus of different follicular and luteal stage was collected from an abattoir in Dhaka city. In the laboratory, the tissue was separated from the caruncular and intercaruncular regions of the cow endometrium. MMPs were isolated from the endometrial tissue by using tissue lysis buffer and gelatin zymography have done. Zymograms of the tissue supernatant showed that MMP-9 and MMP-2 both are differentially expressed in the follicular and luteal group. Total MMP-9 and MMP-2 protein expression were found higher in caruncular tissue in the estrous cycle group. Caruncular tissues have more potential to respond to hormonal-induced MMP secretion. Reproductive hormone estrogen (E2) and progesterone (P4) seems to be influenced the clearance of MMPs since E2 and P4 is the prime hormone of the follicular and luteal stage respectively. In conclusion, hormonal contact of the endometrial tissue during the cyclic stage control the MMP secretion and play regulatory activity of extracellular matrix (ECM) remodeling in endometrium during cyclic stage of cows.

Keywords: cow, endometrium, estrogen, progesterone, MMP

INTRODUCTION

The endometrium, a highly specific layer that surrounds the mostly muscular uterine body, has evolved to sustain the dynamic events necessary to establish as well as maintain pregnancy. Throughout the breeding cycle, the mammalian endometrium undergoes morphological and structural changes. The structural rearrangement of living tissue, which depends on the breakdown and reformation of the extracellular matrix (ECM), is commonly referred to as tissue remodeling of the endometrium (Pinet *et al.,* 2019). ECM is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins. Matrix components bind each other as well as cell adhesion receptors forming a complex network into which cells reside in all tissues and organs (Theocharis *et al.,* 2016). The proteolytic destruction of ECM components is mediated by a number of proteases, although the matrix metalloproteinase (MMPs) family members are the most prevalent. MMPs is a large family of zinc-dependent endopeptidases which include the collagenases, gelatinases, stromelysins, metalloelastases and membrane-type MMPs (Birkedal-Hansen *et al.,* 1993; Nagase and Woessner, 1999). For the window of implantation in ruminants, reproductive uterine remodeling before to implantation is crucial, and it has been revealed that MMP plays a crucial function during this time for a healthy pregnancy (Hashizume, 2007). Structural reformation of bovine endometrium during estrous stage also reported by Arai *et al.* (2013). MMPs have a wide range of possible uses, however the connection between MMPs and tissue remodeling in the ruminant endometrium is still unclear. It was reported that, MMPs are regulating the release or activation of several biological factors thus participating in physiological processes (Loffek *et al.,* 2011). Understanding the function of tissue remodeling in endometrial physiology is made possible by the elucidation of the pattern of MMPs secretion in bovine endometrium, which must be crucial for the development of bovine pregnancy. It was reported that MMPs are spatiotemporally expressed in the uterus of cyclic female of mammals and confirm the structural change for maintain the

[1]Associate Professor, [2] Professor, [3] Assistant Professor, Dept. of Animal Nutrition, Genetics and Breeding, [4] Associate Professor; Dept. of Anatomy, Histology and Physiology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh

pregnancy (Zhang *et al.,* 2007). Balanced secretion of this enzyme is important to maintain the surroundings of the endometrium. Most MMPs act extracellularly and at neutral pH and are released as latent precursors requiring activation by proteases such as plasmin, tryptase and elastase, as well as by other activated MMPs. MMPs can be regulated at a number of levels. Transcription is modified by growth factors, cytokines and steroid hormones, the actions of which are tissue- and cell-type specific and vary among the enzymes (Salamonsen and Woolley,1999) By considering the other studies and their effects, the current work focused on the pattern of expression of MMPs in bovine endometrial tissue to show the likely expressional pattern that regulates tissue remodeling in endometrium during the estrous cycle of cows.

MATERIALS AND METHODS

The experiment was conducted at the Genetics and Breeding laboratory under the Department of Animal Nutrition, Genetics and Breeding at Sher-e-Bangla Agricultural University, Dhaka, Bangladesh.

Preparation of the laboratory

Before starting the experiment, all the necessary electrical power driven or digital equipment were properly installed and checked for good condition. If needed, these were repaired, reinstalled and finally cleaned and sterilized with 70% alcohol. All the reusable equipment were properly washed, dried, covered with aluminum foil, sterilized and finally kept in a cleaned and sterilized chamber until use and same procedure was applied before reuse. All the essential disposable equipment as well as media, chemicals and reagents were made readily available before starting the experiment.

Collection of endometrial tissue from estrous cycle group

Cow uteri and endometrium were collected from Mohammadpur local slaughterhouse of Dhaka city. Total 30 uterus were collected from slaughter house and among them 16 numbers of uterus were found suitable for sample collection and rest are unsuitable due to degraded or missing ovaries. Missing ovaries or reproductive part found difficult to identify the cyclic stage. This unsuitable uterus found 14 in number. The follicular and luteal stage of the estrus cycle was determined by ovarian morphology with consideration of follicular size and corpus luteum (Ireland *et al.,* 1980). During follicle stage, the ovary contains at least one large follicle and a regressed corpus luteum with no vasculature was visualized on its surface, whereas during luteal stage, a corpus luteum is fully formed with vasculature visible around its periphery. Among 16 number of good uterus 5 number was properly identified the cyclic stage by ovarian morphology. Hence, cow uteri from estrus cycle groups, follicular stage (n=5) and luteal stage (n=5), were selected. In the laboratory, reproductive tract of each cow was removed and trimmed of extraneous tissue. The uterus was immersed in 70% ethanol and then washed two times with Phosphate buffer solution (PBS) at 38.5°C. The uterus was opened longitudinally, and caruncular and intercaruncular part of the endometrium were carefully cut from the lamina propria of the endometrium with scissors and transferred in to serum tubes. On the luminal surface of the mature bovine uterus, four irregular rows of oval caruncles can be observed running the length of each horn. These samples were then stored at refrigerator until further processing.

Protein extraction and gelatin zymography

After collection, cow uterine tissues was homogenized using tissue homogenizer. Briefly, put 50–100 mg of caruncular and intercaruncular tissue sample from each follicular and luteal stage tissue to disrupt in the tube separately. Add 250 µl of lysis buffer [20 mM Tris-Hcl (pH 7.5)/150 mM NaCl/2 mM EDTA] in each tube. After fine chopping with sharp scissor insert the pestle into the tube and disrupt the tissue until it appears completely fine in structure. Discard the pestle and add 750 µl of lysis buffer again to increase the volume of the supernatant. After that, vortex it and centrifuged at 15,000 × *g* for 30 sec. Extra amount of tissue supernatant after centrifuge collect in separate tube, marked it and stored in the refrigerator for further use. To determine the enzymatic activity of MMPs, 1 µg of extract protein samples or 10 µl of harvested medium was subjected to Sodium Dodecyl Sulphate-

Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 10% (w/v) acrylamide gel containing gelatin (0.6 mg/ml) under non-reducing condition.

To make gelatin solution, it was heated at 37°C to properly dissolve the gelatin in the water. After making the gel it was run in a1×SDS running buffer [3.03gm Tris/14.4 gm glycine/1gm SDS] with 10µl of loaded sample mixed with sample buffer. Finally, run the electrophoresis machine for protein separation from the sample. The machine was run for 2hr with 80 voltage until the sample buffer reached in the bottom of the gel. After running, the gel was washed with a washing buffer [50 mM Tris-HCl (pH 7.5)/5 mM $CaCl_2$/1 µM $ZnCl_2$/2.5% (v/v) Triton X-100/0.02% (w/v) NaN_3] to remove extra SDS and then incubated at 37°C for 18 hr in an incubation buffer [50 mM Tris-HCl (pH 7.5)/5 mM $CaCl_2$/1 µM $ZnCl_2$]. Thereafter, the gel was stained with staining buffer [0.1% (w/v) coomassie brilliant blue R-250/ 50% (v/v) methanol /20% (v/v) acetic acid] for 1 hr and distained it by using decoloration liquid. Gelatinolytic activity was detected as unstained bands on blue background of the gel. Densitometric analysis was performed using Image J 1.48 software.

Statistical analysis

Single factor analysis of variance (ANOVA) was used to analyze the statistical differences where significances was considered at $P<0.05$. Furthermore, the Student-Newman-Keuls test was used to compare two groups and the differences was considered significant at the level of $P<0.05$.

RESULTS AND DISCUSSION

In the slaughter house most of the animal found non cyclic and their reproductive history was unknown. After slaughtering most of the reproductive part discarded from the edible portion. During slaughtering, reproductive part as well as uterus was harshly treated and it makes removal of the ovary or part of the uterus from the reproductive organ. As a result it makes difficult to identify the cyclic stage of the uterus and damages occur in the uterine tissues. In this study lot of uterus counted as unsuitable to collect sample due to miss handling of the uterus during slaughter in the slaughter house shown in Table 1. and Table 2. After collection the suitable tissue, enzymatic activity of MMP-9 and

Table 1. Total number of cows uterus collected from slaughter house

Total Number of cow uterus collected (n=30)	
No. of uterus suitable for sample collection (n)	No. of uterus not suitable for sample collection (n)
16	14

Table 2. Numbers and parameters of identified cyclic stage uterus.

Types of uterus in cyclic stage	**Number (n)**	**Parameter**
Follicular stage	5	-Large follicle present -Regressed corpus luteum present -No vasculature in ovarian surface
Luteal stage	5	-Small follicle present -Fully formed corpus luteum present -Fully formed vasculature visible in ovarian surface

MMP-2 in cow endometrial tissue were analyzed by gelatin zymography (Fig. 1). Pro-form of MMP-9 was detected in caruncular tissue of both follicular and luteal stage. On the other hand pro-form MMP-9 was only detected in intercaruncular tissue in case of follicular stage but absent in luteal stage. Furthermore, MMP-2 enzymatic activity was detected during all stage of estrous cycle. Both MMP-2 activity of the precursor and mature forms were strongly detected at follicle stage in caruncular and intercaruncular tissue, then the activity become weak at luteal stage. Relative enzymatic activity of

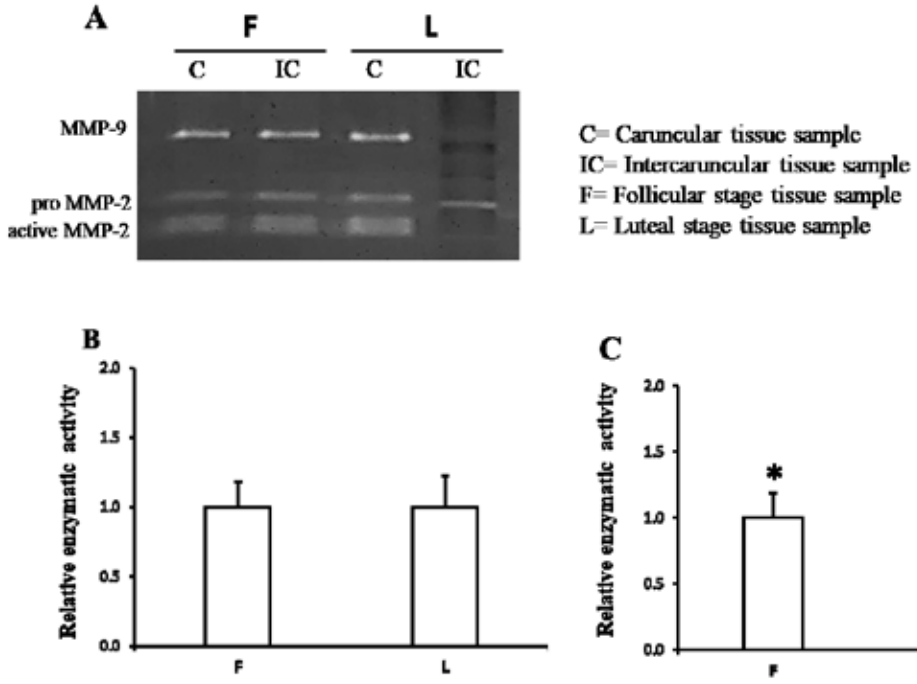

Fig. 1. Expression of MMPs in cow endometrium. Enzymatic activity of MMP-9 and MMP-2 in cow caruncular and intercaruncular tissue during estrous cycle (A). Total enzymatic quntificatioon in caurncular (B) and intercaruncular tissue (C).

caruncular tissue for follicular and luteal stage found no significant ($P>0.05$) difference. Whether as significance differences ($P<0.05$) where observed in intercaruncular tissue in between follicular and luteal stage. It was reported that during estrous cycle, the ECM component collagen type-I and -IV exhibit changes clearly visible both in structures and amounts in bovine endometrium (Boss, 2000). In cattle, the role of ECM remodeling in reproductive process is poorly understood as only few studies were performed in ruminants at the implantation period (Mishra *et al*., 2010). For the process of ECMs remodeling, MMPs is considered as an important mediators (Visse and Nagase, 2003). To unravel the regulatory mechanisms of MMPs in the endometrium, in vitro culture models were typically used. Previous reports suggested that the expression of several MMPs was controlled by steroid hormone (Vassilev *et al.,* 2005) and by soluble local regulators such as hormone and other factors (Pretto *et al.,* 2008). In this study, tissue were collected from slaughter house uterus of different estrous group where E2 and P4 was key regulator of this stage which regulate different biomolecule including MMP. The expression of MMP-9 and MMP-2 has also been reported within the uterus of domestic animals including bovine (Hashizume *et al.,* 2003), goats (Uekita *et al.,* 2001) and sheep (Salamonsen *et al.,* 1995) respectively. Although, the information in this regard is very limited. It was reported that, MMP-2 were secreted by cultured ovine endometrial stromal, but not epithelial cells whereas MMP-2 production is constitutive (Salamonsen *et al.,* 1995). Our report also support the previous statement where MMP-2 consecutively secrete follicular and luteal tissue. Although the patterns of specific MMP expression that are regulated by estrogens or progesterone are not yet fully established, since lot of factor is involved in in vivo condition. Accumulated research suggests that ovarian steroids affect the gene expression of the MMP system in the uterus. P4 appears to down-regulate some MMPs in the uterus.Though MMP-9 and MM P-2 secrete in luteal stage but in this study MMP-9 and MMP-2 seems to be less secret in luteal stage intercaruncular tissue. It might be due to P4 effects. Additionally,

findings of MMPs expression in cow endometrial tissues in this study also supported this suggestion because enzymatic activity of MMP-9 was hardly detected in all stages. The cellular difference of caruncular and intercaruncular area is well documented. Caruncular and intercaruncular area are covered by dense stroma and large number of branched glands respectively. This structural distinction also indicate the functional inequality of the endometrium also. The difference of enzymatic secretion of caruncular and intercaruncular region confirm the cellular dissimilarity of the specific region of the endometrium. The alteration of the MMP-9 and MMP-2 secretion may also regulated by the unknown cell secretory factor. However, from this data it is assumed that after synthesis of MMP-9 and MMP-2 it does not release in the tissue environment immediately, somehow it stored in the tissue which can be detected through zymography otherwise it cannot be detected after long time of tissue processing. These results suggested that other factors except E2 and P4 might have another effects on the expression of MMP-9 and MMP-2 mRNA in cow endometrium. Since different form of MMP-9 and MMP-2 are differentially expressed in the tissue; it is possible to predict that there activation from pro to mature form is regulated by some inherent factor from the tissue cells which regulate the activation for specific function of the endometrium. In summary, this study revealed that hormonal influence regulated the MMPs clearance and that released MMPs induced ECMs remodeling in cow endometrium.

CONCLUSION

In summary, this study revealed that steroid hormone regulated MMPs clearance and that released MMPs induced ECMs remodeling in cow endometrium. Furthermore, caruncular and intercaruncular site of the endometrium differentially expressed the MMPs. Since placentation is occurred in caruncular site hence MMPs role is to remodel the implantation site for successful implantation and this remodeling occurs continuously in each cycle of cow. However, to explore more in MMPs expression; mimic physiological condition is necessary. That's why spheroids are more suitable for analyzing the regulatory mechanisms of ECMs remodeling and the related factors in the cow endometrium compared with monolayer cell culture models should applied in future.

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Compliance with ethical Statements
Conflict of interest: The authors declare that they have no conflict of interest.

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