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INVESTIGATION OF OVARIAN MATRIX METALLOPROTEINASE (MMP) 1, 2 AND TOTAL NITRIC OXIDE (NO) IN INFERTILE DAIRY COWS

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ABSTRACT

The object of this study was to investigate changes of total NO, ovarian MMP1 and MMP2 correlated with LH and E2 in infertile dairy cows. 40 Holstein cows included 21 infertile cows and 19 normal cows during their estrous phase to limit as possible as the hormones fluctuation were concerned. Blood were used for measurement of LH, E2 and NO. The ovarian tissues were used for evaluation of MMP-1 and MMP-2 with Real-Time PCR and Immunohistochemistry. LH, E2 and NO results were 12.25 ± 0.32, 49.70 ± 8.08 and 9.76 ± 1.10 respectively in normal cows, but in infertile cows were 17.60 ± 0.65, 82.53 ± 6.49 and 15.53 ± 1.42 respectively, the difference of these hormones decreased significant in the infertile cows $P<0.05$. PCR results showed the expressions of MMP1 and MMP2 in ovarian tissue were lower in infertile cows, compared with fertile cows ($P<0.05$), These findings were approved by the immunohistochemical localizations of MMP-1 and MMP-2 in the different part of ovarian tissues or cells including granulosa and theca cells of preovulatory follicle, epithelial follicular cells of small follicles, stromal cells and endothelial cells of blood vessels. This data verified that infertile cows have poor ovarian activity and follicular development. Decreases of expression of MMP1 and MMP2, which are essential for ovulation, may be considered to be important marker of disrupted ovarian function and also poorly follicular development . Moreover, there was a positive correlation between NO level and improper follicular development. In conclusion, it was determined that MMP1 and MMP2 are factors affecting fertility in cows correlated with NO and hormonal profile.

KEYWORDS: Matrix MetalloProteinases (MMPs), Nitric Oxide (NO), LH, E2, Infertility, Ovarian Tissue, Cows

1. INTRODUCTION

Reproductive performance is one of the most important factor affecting dairy farm profitability, because it directly or indirectly influences the yield of milk, reproductive culling rate and the cost for breeding and calf sales (21). Dairy cows should calve one time every year to maximize economic efficiency, but their reproductive function had declined obviously in the past twenty years (20). Cows that have been highly selected for milk production in recent decades have suffered a decline in cow fertility, fertility is a multi-factorial trait and its deterioration has been caused by a network of genetic, environmental and managerial factors and their complex interactions make it difficult to determine the exact reason for this decline (35). The ovaries play the key roles in reproduction and any impairment in their functions can results in either sterility or infertility (1).

Ovaries undergo continuous tissue remodeling during follicular growth, maturation and ovulation. A family of proteins known as the matrix metalloproteinases (MMPs) play an important role in this process by clearing the way for new growth through cleaving ovarian tissue components, releasing growth factors, and contributing in the degradation of the extracellular matrix (36). MMPs is a family of zinc-dependent endopeptidases with proteolytic activities against several components of the extracellular matrix (ECM), the function of the active enzyme is to digest the ECM (12). Type I and III collagen-rich ECM in the theca external and tunica albuginea layers of the preovulatory follicle wall, and there is evidence that selective collagen degradation occurs at the apex of the follicle (8, 17). Bakke et al., (4), demonstrated the MMP-1 immunoreactivity was localized to both the granulosal and the thecal layers of bovine preovulatory follicles relative to GnRH injection, with minimal specific expression detected in the adjacent ovarian stroma. Another study explained MMP-2 increased significantly in the bovine follicular fluid relative to enlargement of follicles size reach the maximum in the preovulatory follicle of normal cows (13).

Nitric oxide (NO) has been well recognized as an intra- and intercellular modulator in many biological processes, including vasodilation, maintenance of endothelial cell barrier function and control of apoptosis (29). It plays a key role in ovarian physiology, including follicular development, ovulation, CL function, and ovarian vasodilatation (2, 32). Nitric oxide is synthesized from l-arginine, either by a constitutive calcium-dependent neuronal NO synthase (nNOS) and endothelial NOS (eNOS), or by a pro-inflammatory cytokine-inducible NOS (iNOS) in vivo (16). It is well known that NO is a labile and diffusible molecule, forming stable oxidized metabolites (nitrite/nitrate), which are present in many biological

processes (26). In this regard, NO is present in the follicular fluid (FF) of cattle, and it is produced by granulosa cells in culture; the most active granulosa cells producing NO are those from the small follicles (5, 19). The object of this study was to investigate changes of total NO, ovarian MMP1 and MMP2 correlated with LH and E2 in infertile dairy cows.

2. Material and methods

2.1. Animals and samples

This work was carried out in Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, during period from May to November 2012. 40 Holstein cows included 21 infertile cows and 19 normal cows at the estrous phase to limit as possible as the hormones fluctuation were participated in this work . The cows were taken from three farms as followings farms: 13 cows from Huazhuang dairy farm of Lanzhou (6 infertile and 7 normal), 14 cows from Qinwangchuan dairy farm of Lanzhou (8 infertile and 6 normal), and 13 cows from Zhenxin dairy farm of Yinchuan (8 infertile and 5 normal). Infertile cows were determined according to birth space. The average of the birth space in infertile cows was 8 months, these cows were mounted by artificial insemination 4–5 times according to the farm records and they were 7.1 years old. The average of the birth space in normal cows was 3.2 months and the average age was 5.2 years. Jugular blood samples were collected and transferred into ice box, the serum was separated in the laboratory by centrifugation at 300 rpm/min and stored at -20 ℃ until measuring the LH, E2 and NO. Ovaries of 6 cows were collected from abattoir (n=3 of each group), immediately cut the ovaries into small pieces, some of them were snap frozen in liquid N_2 and stored at -80 ℃ until RNA extraction, other ovarian tissue pieces were fixed in 10% formalin for the immune-histochemical detection of MMP-1 and MMP-2.

2.2. Measurement of Luteinizing hormone (LH) and Estradiol (E2)

The measurement of (LH) and E2 were done by ELISA method using Bovine LH and Bovine E2 kits produced by Abnova Company, Taiwan. The assay procedure was done according to the manufacturer's instructions of each kit. The results were recorded after construction a standard curve of each hormone.

2.3. Measurement of the Nitric oxide (NO)

Nitric oxide in the present study was measured by Greiss reagent method using a kit presented by Promega Company (USA). The assay protocol was done according to the manufacture's instructions; briefly, prepare the standard dilutions to establish the standard curve, allow the sulfanilamide solution and *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED) solution to equilibrate to room temperature (15 – 30 min). Add 50 µl of each sample serum to each wells

in duplicate. Using the multichannel pipette, dispense 50µl of the sulfanilamide to each well. Incubate 5 – 10 minutes at room temperature, protected from light. Dispense 50 µl of the NED solution then incubate at room temperature for 5 – 10 minutes also protected from the light, a purple/magenta color will begin to form immediately. Read the absorbance in the plate reader at 535 nm; calculate nitrite concentrations of the samples after construction the standard reference curve.

2.4. Total RNA extraction

The total cellular RNA extraction of the ovarian tissue was done according to manufacture's instructions of total RNA extraction kit presented by Omega Biotech (USA). 100 mg ovarian tissue was homogenized briefly by liquid N_2 in the ceramic mortar, when the liquid N_2 has completely evaporated, added 1mL of RNA-Solv reagent, then transfered to a clean 1.5 mL tube. After been incubating for 3 min, added 0.2 mL of chloroform, shaked vigorously for 15 seconds, then incubated on ice for 10 minutes. After the tube was centrifuged at 12 000 rpm for 10 min, no more than 80% of aqueous part was transferred into a clean tube and added 1/3 volume of absolute ethanol. After been vortexed, 700 µL of mixture was transfered into Hibind RNA Column, centrifuged at 10 000 rpm for 60 seconds at room temperature. Then, the column was moved into a new tube and added 300 µL RNA washing buffer I, then centrifuged same as above, this step repeated 2 times, discarded the tube content and added 500 µL RNA washing buffer II diluted with ethanol, centrifuged same as the last step, transferred the column into new tube and added 50 µL DEPC water, then centrifuged at full speed for 1 minute. The amount of total RNA was determined using nanodrop spectrophotometer (Thermo Scientific, USA) at 260 nm. RNA was reversed transcribed into cDNA with random primers (prime Script RT reagent Kit – TAKARA Biotechnology Co. China).

2.5. Real-time PCR

The quantity of gene expression of MMP-1 and MMP-2 were done by real-time RCR using SYBR green assay (TAKARA biotech.Co. China). Primers pairs of MMP-1 and MMP-2 were designed using Primer Primier 5.0 software depending on the bovine sequence of MMP-1 and MMP-2 (Table 1). PCR reaction (using BIO-RAD multicolor Real Time- PCR, USA) was denaturation of DNA at 95 for 30 seconds one cycle, and amplifying and quantifying at 95 for 5 seconds and 60 for 30 seconds repeated 40 times. The relative mRNA expression of MMP-1 and MMP-2 were normalized to GADPH and melting curve of each gene.

Table 1. Oligonucleotide primers used for Real Time – PCR.

Gene	Primer	Sequence	Length (bp)
MMP-1	Forward	TGTGTCGTGTTAGTCGCTCAGTC	221
(NM-174112)	Reverse	AACCCGCTCCAGTATTCTTGC	
MMP-2	Forward	CGAACGCCATCCCTGATAACC	197
(NM_174745)	Reverse	TGCTTCCAACTTCACGCTCTT	
GADPH	Forward	GCAAGTTCAACGGCACAGTCA	205
(NM_001034034)	Reverse	TTGGTTCACGCCCATCACA	

2.6. Immunohistochemistry

The immunohistochemical demonstration of MMP-1 and MMP-2 was done based on procedure used by Riley et al., (25) with slightly modifications. MMP-1, 2 (primary antibodies) were purchased from (lifespan biosciences, USA), Streptavidin-biotin-peroxidase compound (SABC), 3,3'-diaminobenzidine (DAB) appears brown, Biotinylated goat anti rabbit IgG and bovine serum albumin (BSA) were purchased from (Boster company Wuhan-china). Staining protocol in Brief, ovarian tissues were fixed in 10% formalin as mentioned, dehydrated in ethanol, embedded in paraffin wax, and sectioned at 5 µm. Staining process started by dewax in xylene, rehydration in ethanol, inactivation endogenous enzymes with 3% H_2O_2 at room temperature for 30 min. Washing in distilled water, adding 5% BSA confining liquid at room temperature rest for 20 min, throwing away excess liquid. Incubating the sections in the primary antibodies of MMP-1 and MMP-2 for 17 hours at 4 ℃, washing in PBS (pH 7.2-7.6) again. Adding biotinylated goat anti rabbit IgG (secondary antibody), reacting at 20-37 ℃ for 20 min. Washing in PBS and adding SABC, reacting at 20-37 ℃ for 20 min. Washing it and then DAB chromogenic method staining for 20 min. Counter staining with harris Hematoxylin mildly for 1 min, then differentiating with acid-alcohol, dehydration, transparentizing, mounting the piece.

2.7. Statistical Analysis

Real-time PCR was run in triplicate, quantitative value were expressed as MEAN ± S.D. The results of hormones and nitric oxide were presented as MEAN ± SEM. Independent t-test was used to compare the difference between the normal and infertile cows. A value $P<0.05$ was considered to significant. For immunostaining of tissue sections was assessed by semi-quantity of the ovaries using + and – symbols, as a measure of the intensity. + indicate pale staining, ++ indicate marked staining, +++ signifies intense immunostaining, while score – means that is no positive stain (27).

3. Results

3.1. LH, E2 and Nitric oxide results

In general, the results of LH, E2 and NO (Table 2) revealed low levels in infertile cows compared with normal cows. LH was lower levels in the infertile cows than the normal cows, the analysis of these data showed significant difference ($P<0.05$). The estradiol was markedly decreased in infertile cows ($P<0.05$). The result of Nitric oxide (NO) level of the normal cows was higher than that of infertile cows, this difference was statistically significant $P<0.05$.

Table 2. Illustrate the results of LH, E2, and Nitric oxide levels of infertile and normal cows (mean ± SEM)

Parameter	Infertile cows	Normal cows
LH (ng/ml)*	12.25 ± 0.32	17.60 ± 0.65
E2 (pg/ml)*	49.70 ± 8.08	82.53 ± 6.49
Nitric oxide (uM)	9.76 ± 1.10	15.53 ± 1.42

*Differences between infertile and fertile cows is statistically significant for LH, E2 and NO parameters- (P<0.05).

3.2. Gene expression of MMP-1 and MMP-2

The quantity expression of MMP-1 and MMP-2 in the ovarian tissue of normal cow and infertile cows were normalized to GADPH of bovine sequence. MMP-1 record was 0.9 ± 0.08 in the normal cows, while significant decrease *(P< 0.05)* was observed with 0.54 ± 0.07 in the infertile cows. The mRNA expression of MMP-2 was 1.24 ± 0.071 in normal cows and 0.72 ± 0.076 in infertile cows, respectively. This decrease of MMP-2 expression was statistically significant *(P< 0.05)* as showed in (Figure1).

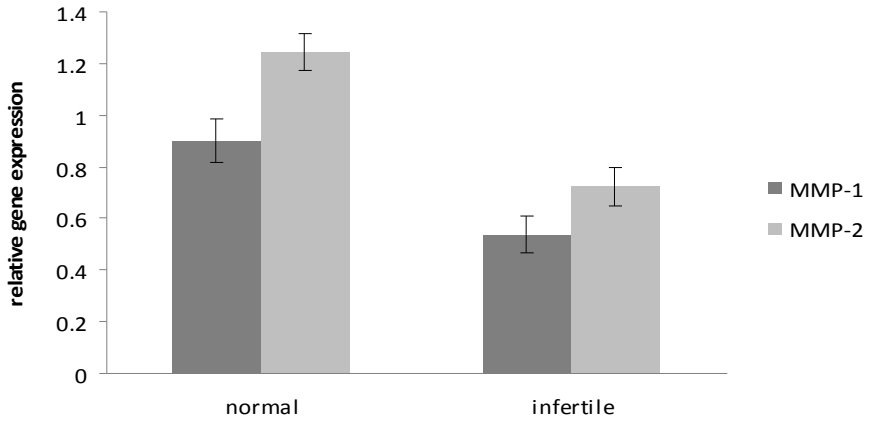

Figure 1. Quantitative expressions of MMP-1 and MMP-2 in the ovaries of infertile and normal cows measured by real time - PCR showed a significant decrease of both genes in ovaries of infertile cows compare with normal cows $P< 0.05$.

2.3. MMPs distribution

The immunohistochemical results revealed the specific expression pattern of MMP-1 and MMP-2 in the ovary of both normal and infertile cows. The results were summarized in table 3 which showed semi-quantitative expression representative by +/- symbols. Immunolocalization of MMP-1 in ovaries of normal cows shown in Figures 2A – E. MMP-1 expressed strongly in the cytoplasm of granulosa and theca cells of pre-ovulatory follicle as in Figure 2A. Conversely, MMP-1 expressed slightly in the granulosa and theca cells of pre-ovulatory follicle shown in Figure 3A. The follicular epithelial cells of primordial and primary follicles expressed MMP-1 clearly as in Figure 2 (C and D), while the results recorded a decrease expression in the infertile cows primary ovaries (Figure 3C) and primordial follicles (Figure 3D). MMP-1 expression appeared in epithelial cells of secondary follicles of normal ovaries, but this expression clearly decreased in ovaries of infertile cows (Figure 2 E and Figure 3C). Ovarian stromal and endothelial cells of blood vessels in the ovarian stroma (Figure 2 B and E) expressed MMP-1 strongly in their cytoplasm, in contrast, these structures showed faint expression of MMP-1 in their cytoplasm (Figure 3 B, C).

Specific positive immunostaining for MMP-2 appeared in ovaries of normal cows rather than infertile cows. In ovaries of normal cows, MMP-2 revealed consistent increase throughout follicular developing stages. In mature follicles of normal cows, MMP-2 showed predominant expression in the granulosa and theca cells. It showed a band like structure in the granulosa layer with under low magnification (Figure 4A and Figure 4B). But mature follicle in ovaries of infertile cows showed strongly decrease expression in theca and granulosa with high magnification (Figure 5A,B). MMP-2 expression appeared in the follicular epithelial cells of primordial follicle and endothelial cells of blood vessels in their cytoplasm (Figure 4C). The expression of MMP-2 expressed strongly on the cuboidal follicular epithelial cells of primary follicles shown in Figure 4D as a dark brown color, while in ovaries of infertile cows showed decrease expression of MMP-2 clearly in the epithelial cells of primordial and primary follicles as in Figure 5C, D, respectively. Secondary follicles also expressed MMP-2 in the follicular cells of normal cows, but this expression decrease in infertile cows (Figure 4E and Figure 5E).

Table 3. Illustrate the semi-quantitative immuno-localization of MMP-1 and MMP-2 in the ovaries of normal and infertile cows.

Structure	MMP-1 Normal / Infertile		MMP-2 Normal / Infertile	
Granulosa cells- mature follicle	+++	++	+++	+
Theca cells - Mature follicle	++	+	+++	++
Primordial follicle	++	+	++	+
Primary follicle	++	+	+++	++
Secondary follicle	++	+	++	+
Interstitial & endothelial cells	++	+	++	+

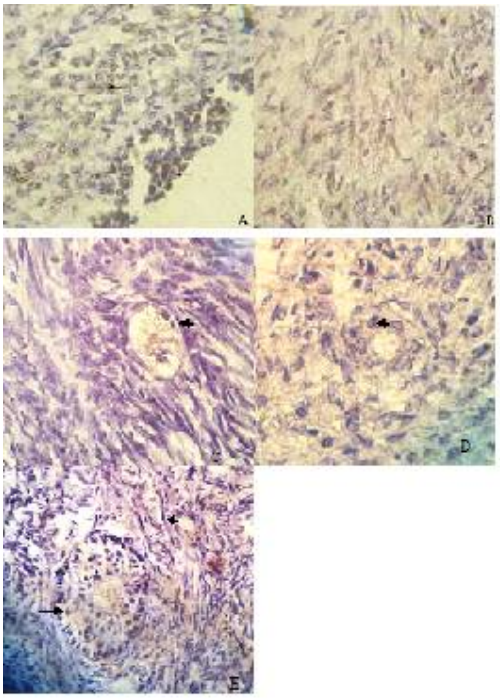

Figure 2. Immunohistochemical sections showing MMP-1 expression in ovary of normal cows. (A) granulosa and theca cells of the mature follicle. (B) The interstitial cells of ovarian stroma. (C) epithelial follicular cells of the primordial follicle (D) Epithelial follicular cells of primary follicle (E) Secondary follicle and endothelial cells of blood vessel, (magnification of A. is X200; B,C and D are X (1000)3.1; E is X(400)2.8. Arrows refer to cells expressed MMP-1.

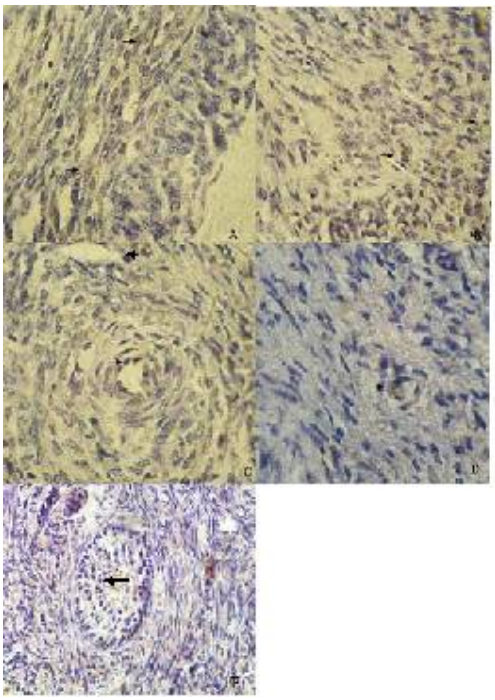

Figure 3. Immunohistochemical sections showing a decrease expression of MMP1 in the ovary of infertile cows. (A) The granulosa and theca cells of the mature follicle, (B) Interstitial cells of ovarian stroma. (C) Primary follicle and endothelial cells of blood vessels. (D) MMP-1 expression in the epithelial cells of primordial follicle. (E) Slightly expression in the epithelial follicular cells of secondary, (magnification of A, B,C,D. is X(1000)2.8, magnification of E is X(400). Arrows refer to cells expressed MMP-1.

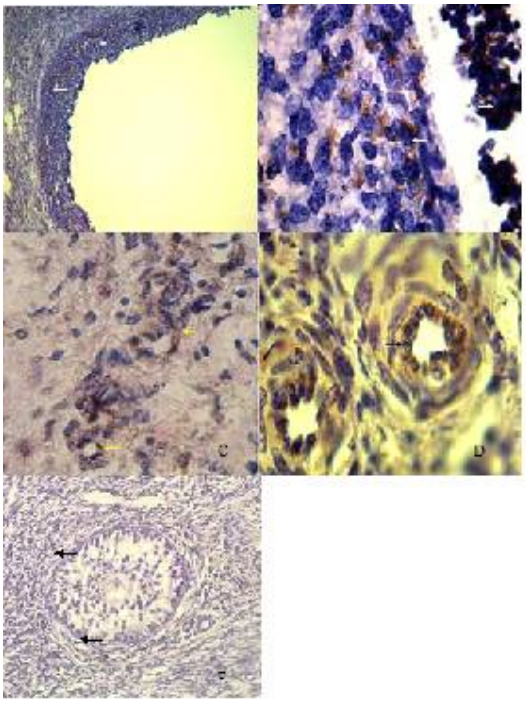

Figure 4. Immunohistochemical Showing the expression of MMP2 on ovary of normal cows, the figure illustrates intensive expression in the different ovarian parts. (A and B) showed theca cells and the granulosa cells of the mature follicle. (C) The epithelial follicular cells of primordial follicle, endothelial cells of blood vessels and the interstitial stromal cells (D) Epithelial follicular cells of primary follicle. (E) Secondary follicle. Arrows refer to cells expressed MMP-2 (magnification of A. is X200; B,C and D are X (1000)3.1; E is X(400)3.1.

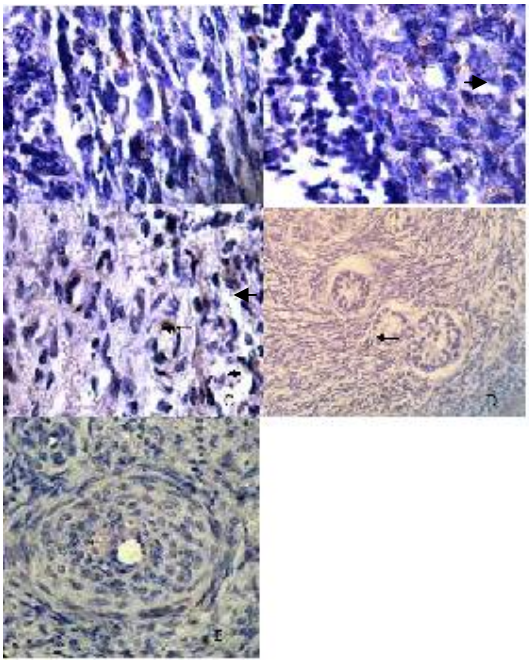

Fig. 5. Immunohistochemical sections showing expression of MMP2 on ovary of infertile cows. (A and B) showing MMP-2 faint expression in the granulosa cells and theca cells of the mature follicle. (C) MMP-2 expression in the ovarian stroma cells, endothelial cells and follicular epithelial cells of primordial follicle. (D) the epithelial follicular cells of primary follicle. (E) sharply decrease MMP-1 expression in the secondary follicle; Arrows refer to cells expressed MMP-2, magnification X(1000)3.1.

4. Discussion

This study is the first to demonstrate the quantity and immuno-localization of MMP-1 and MMP-2 in the field of infertility in dairy cows. The aim of this study was to evaluate the effects of infertility on the ovarian activity to express these molecules assisted to LH, E2 and NO records.

E2 and NO levels were low at estrous phase of cycle in those infertile cows compared with normal cows. These findings indicated that the poorly follicular growth and maturation in the

ovary may lead to decrease of LH surge, because few of the feedback signal is represented by E2 for LH release and the secretion of LH surge from pituitary gland may have been suppressed by low level of NO or vice versa.

LH levels decreased in the infertile cows due to insufficient feedback to secret LH from pituitary. Plasma E2 showed a clear increase during the late follicular phase and a few preovulatory follicles were observed in the ovaries, which supported the hypothesis that E2 is a chemical signal of follicular maturation in the ovary in many mammals (33). The low level E2 in infertile cows suggested there is only few numbers of dominant and subordinate follicles in the ovary. E2 is an ovarian marker with feedback regulation on FSH and LH secretion and it could reflect the development and maturation of dominant follicles (7). Adequate LH secretion is needed to support follicular and oocyte maturation and also subsequent ovulation of a dominant follicle (28).

Endothelial NO synthase was detected in granulosa and theca cells, as well as in blood vessels from primordial to antral follicles (34), also it has been suggested that NO is involved in follicular maturation and ovulation in women and in vasodilation of follicular vessels around the time of ovulation in rats (18). The results showed there is a certain relationship between NO level and the development of the follicles. This evidence approved that the lower level of NO in infertile cows due to the less number of developing follicles, also suggested that lower level of LH in infertile cows due to not only the E2 insufficient feedback but also the low level of NO.

The previous findings indicated that the inhibition of NO synthesis could lead to decrease the plasma LH concentration and abolish LH pulse. LH is brought about by pulsatile release of LHRH that is driven in turn by pulsatile release of norepinephrine; The latter acts on α1-adrenergic receptors to induce LHRH release from terminals of LHRH-secreting neurons in juxtaposition to hypophyseal portal capillaries in the median eminence. NO block leads completely the norepinephrine to induce LHRH release and decrease LH secretion and LH pulsatile (24).

Many previous worked described the role and localization of MMPs of bovine follicles and in the bovine ovaries but they were done on the normal cows (4, 13), the present study focus to explain these molecules in infertile cows compared to normal cows. MMPs are secreted as latent proenzymes which are proteolytically cleaved to their active forms (38). Bovine antral follicles compose of several cell types including granulosa and theca cells, fibroblasts and endothelial cells. ECM influences basic cellular processes such as proliferation,

differentiation, migration and adhesion (22). The gonadotropin surge induced regulation of specific MMP and plasminogen activator (PA) system components (14). A previous study mentioned the MMP-2, MMP-9 and other ECM remodeling proteins is secreted in the estrogenic granulosa cells (30), which may explain increasing E2 secretion accompanied with increase the secretion of MMP-2. In addition, the estrogen is relative to the maturation and development of the follicle on the ovary so that MMP-2 should increase throughout follicles growth.

MMP-2 is a gelatinase with an ability to degrade and denature basement membrane collagens (37). In the ovary, they may permit turnover and reconstruction of the follicle wall at the time of growth (23) and ovulation (31), and facilitate the remodeling of tissue during corpus luteum formation and development (15). It was reported the expression of MMP genes was up-regulated in the theca cells of large preovulatory and ovulating follicles (11). In present study, the expression of MMP-2 was decreased throughout follicular stages (primary, secondary and pre-ovulatory follicles), which showed there was improper growth and development of follicles in those infertile cows. This is supported by the evidence of the MMPs location and expression of in the ovarian stroma cells which may indicate a possible role in the migration of the follicle to ovulation fossa in equine (25). Accordingly, the fluctuation in proMMP-2 activity in the various sizes of follicles could be associated with these continuous wave-like follicular growth patterns observed cows, however, this needs to be verified. Folliculogenesis involves extensive tissue growth, cell migration and angiogenesis (13), also approved MMP-2 increased as follicular size is progress. Consequently, amount of MMPs should be increasing in ovarian stroma during folliculogenesis and so decrease of amount of MMPs in ovarian tissue may be indicator of improper growth of follicles. Also collagenolytic enzymes like MMPs, are thought to play a vital role in ovulation of rats, macaques and equine (9, 6, 25). These enzymes include the fibrillar collagenases such as MMP-1 which can break down the fibrillar collagen forms that confer much of the structural integrity to the ovarian stroma, this explained that MMP-1 was expressed in theca and granulosa cells of mature follicle. The gelatinase activity was increased in the rat preovulatory follicle in response to LH surge (10). MMPs were detected in similar amount at different phases of follicular growth and they are present throughout follicular growth specially (3). Bakke et al., (4) revealed the expression of MMP-1 and MMP-13 in the thecal layer of bovine preovulatory follicle and increase after GNRH sure consistent with a potential role of these enzymes in collagenolysis and the ovulation process. these findings above may indicated the

MMPs are possibly as a marker of follicular growth, they are bi-signal to control the secretion of LH surge and it refer to the stage of follicular growth and feedback by LH.

The expression of MMP-1 and MMP-2 in blood vessels is a signal of growing rate and the functional activity of the ovary and follicles, which was expressed much abundant in the normal cow ovary than in the infertile cows in current study. The previous studies showed the complex process from pre-existing blood vessels to a new vessel requires the production of MMPs, which degrade the basement membrane surrounding the endothelial cells, furthermore, this alteration in the ECM leads to endothelial cells proliferation and migration, as a result, a new vessel is extended (38). Additionally, follicular wall enlarged and developed when the ECM was broken down by MMPs so that the improper and insufficient expression of MMPs in ovaries may indicated insufficient growth and hormones disturbance in the infertile cows.

In conclusion, the results suggested that cows suffered from infertility have a poor ovarian activity and poorly follicular development. The decrease of MMP-1 and MMP-2 expressions indicated that the normal ovarian activities could be interrupted and these MMPs could be considered to be effective marker of the ovarian functions and follicles development. In infertile cows, there was a positive relationship between NO level decline and the case of improper follicular development and hormonal disturbance in current study.

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