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Expression of Matrix Metalloproteinase-2 mRNA in the Chicken Ovary in Relation to Follicle Remodelling*

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In the mammalian ovary, matrix metalloproteinase-2 (MMP-2) is thought to be involved in the regulated turnover of the extracellular matrix during the process of follicle development, ovulation and regression. Information concerning the role of MMP-2 in the chicken ovary is scarce. The present investigation was undertaken to determine the distribution of MMP-2 mRNA within the laying hen ovary as well as to define whether the expression of MMP-2 mRNA changes during the ovulatory cycle. Expression of MMP-2 mRNA in ovarian compartments was determined by RT-PCR analysis at two stages of the ovulatory cycle, i.e. at 22 h and 3 h before ovulation. MMP-2 mRNA was detected in the ovarian stroma and follicles: white, yellowish, small yellow, the largest preovulatory (F3-F1) and postovulatory (P1-P5). In the wall of preovulatory follicles MMP-2 mRNA was found in the theca and granulosa layers. Within the ovary the relative expression of MMP-2 mRNA depended on follicular size, the layer of the follicular wall, the stage of the ovulatory cycle and the degree of regression in the postovulatory follicles. The results obtained indicate that in the chicken ovary MMP-2 may take part in the extracellular matrix remodelling required for follicle growth, development and regression.

Key words: MMP-2, RT-PCR, remodelling, ovary, chicken.

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In the domestic hen, the functionally mature ovary contains follicles at different developmental stages. The most numerous is a group of small prehierarchical follicles classified according to their size into: cortical follicles (<1 mm in diameter) embedded in the ovarian stroma, white follicles (>1-4 mm) and yellowish follicles (>4-8 mm). The next group comprises yellow follicles (>8-36 mm) arranged into a preovulatory hierarchy in which the largest follicle (F1) is the most mature and is first in line to ovulate, the second largest (F2) will ovulate on the following day, and so forth. After ovulation of the F_1 follicle, which occurs every 24-26 h, except for the day when ovulation does not occur, each less mature follicle is moved up one position in the hierarchy and one prehierarchical follicle is selected into the preovulatory hierarchy (BAHR & JOHNSON 1984). Moreover, in the ovary several postovulatory follicles exist which rapidly begin to regress shortly after ovulation (SUNDARESAN *et al*. 2008).

During follicular development, ovulation and subsequent regression, multihormonally controlled extensive tissue remodeling and angiogenesis take place in the ovary. Such processes require cyclic turnover of the extracellular matrix (ECM) components. The ECM provides the tissue specific scaffolding to which cells attach and which modulates activities of cells through cellular surface receptors. Therefore, the ECM seems to be essential for normal development and regression of the follicles, as it influences basic cellular processes such as proliferation, differentiation, adhesion, and cell death (MATRISIAN 1990; PRICE *et al*. 1997; for a review see NY *et al*. 2002).

Due to the complex composition of the ECM, various proteases with various substrate specificities are needed for ECM remodeling and degradation. Numerous studies have established that the matrix metalloproteinase (MMP) system plays a key role in matrix turnover during remodeling of several type of tissues, including reproductive

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ones, and thus is important in many reproductive processes such as follicle development, ovulation, and atresia (HULBOY *et al*. 1997; for a review see SMITH *et al.* 1999, 2002; NY *et al.* 2002; CURRY & OSTEEN 2003).

The MMPs are a growing family of zinc-dependent enzymes that can be divided into subgroups based on domain organization and substrate preference, i.e. collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMPs) and others (SMITH *et al.* 1999; PAGE-MCCAW *et al.* 2007). The MMP family members, especially MMP-2 (gelatinase A), are known to be produced in the mammalian ovary and participation of these enzymes in ovarian function has been strongly suggested (HULBOY *et al*. 1997; SMITH *et al*. 2002; NY *et al*. 2002; BASINI *et al.* 2011).

Gelatinase A is the most widespread MMP and has substrate specificity for a broad spectrum of ECM molecules such as collagen types IV, V, VII, X, XI, fibronectin, laminin, elastin, plasminogen and gelatins, and also for many non-ECM molecules including cytokines, growth factors and their binding proteins (STAMENKOVIC 2003). Therefore, MMP-2 is suggested to play a key role in the degradation of the basement membrane (as it consists mainly of collagen IV) which separates the granulosal and thecal layers in ovarian follicles (LIU *et al.* 1998; NY *et al.* 2002).

Information concerning the expression and role of MMPs in the avian ovary is scarce. NAKAJO *et al*. (1973) suggested that proteolytic enzymes such as collagenase play a role in mechanisms of follicular rupture in the hen ovary. ASEM *et al*. (2000) showed the presence of several MMPs in the basal lamina of chicken preovulatory follicles. Accordingly, the present study was undertaken in order to examine the distribution of MMP-2 mRNA in the chicken ovary. Additionally, we attempt to define whether the expression of MMP-2 mRNA in ovarian compartments changes during the ovulatory cycle.

Material and Methods

Birds

The experiment was carried out in accordance with the research protocol approved by the Local Animal Ethics Committee (No. 50/OP/2004). Hy-Line laying hens (layer strain) purchased from the commercial farm Drobeco (Palowice, Poland) were caged individually under a photoperiod of 14L:10D. They had free access to commercial food and water. Based on recording of oviposition time, cloacal palpation and autopsy, it was found that ovulation occurred about 5 min after oviposition of the previous egg in the series. At the age of 40 weeks the chickens (n=6) were killed by decapitation approximately 22 h before the predicted time of ovulation of the largest preovulatory follicle (F1). The ovaries were collected, placed on ice and the following ovarian compartments were isolated: the stroma with cortical follicles <1 mm in diameter (STR), white follicles (>1-4 mm; WF), yellowish follicles (>4-8 mm; YF), small yellow follicles (>8-12 mm; SYF), 3 of the largest yellow preovulatory follicles F3-F1 (F3<F2<F1) and 5 postovulatory follicles P1-P5 (P1>P2>P3>P4>P5). From the preovulatory follicles, the theca (T) and granulosa (G) layers were separated. Additionally, the follicles, except the postovulatory ones, were isolated from the ovaries at the stage of 3 h before ovulation. Tissues were immediately placed into RNAlater and stored until total RNA extraction.

Chemicals

The following chemicals were used: TRI-reagent (MRC, Inc., Cincinnati, OH, USA), dNTP mix, $MgCl_2$, Pol Taq DNA Polymerase, buffers, molecular weight marker – 100 bp DNA ladder (Fermentas, Vilnius, Lithuania), primers (IBB, Warszawa, Poland), High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). All other reagents were obtained from ICN Biomedicals (Aurora, IL, USA), Sigma (St. Louis, MO, USA) or POCH (Gliwice, Poland).

Total RNA isolation and RT-PCR analysis

Total RNA was isolated from collected tissues with the TRI Reagent, in agreement with the included protocol. Total RNAs (2 μg) from each tissue were reverse-transcribed with MuLV Reverse Transcriptase and Random Hexamers Primers. Non transcribed tissue RNA (reverse transcriptase omitted) was used as a negative control. Reverse transcribed cDNA (1 μl) was amplified in a Thermocycler Gradient (Eppendorf, Germany) in 12.5 μl of reaction mixture containing 1.25 μl of buffer (100 mmol Tris-HCl, pH 8.8, 500 mmol KCl, 0.8% Nonidet P40), 0.312 unit pol Taq DNA polymerase, 0.2 μmol sense and antisense primers, 0.2 mmol each dNTP, 1.5 mmol $MgCl_2$, and water. The oligonucleotide primer pairs for chicken MMP-2 and gliceraldehyde-3-phosphate dehydrogenase (GADPH) are given in Table 1. After the initial denaturation at 94^{o}C for 2 min for MMP-2 or 5 min for GADPH, amplifications were conducted as shown in Table 1. Amplifications were completed with an additional extension at 72^{o}C for 2 min for MMP-2 or 7 min for GADPH. Negative control (water) was included in all reactions. All PCR products were electrophoresed in a

Table 1

Characteristic of primers and PCR conditions used in this study

Gene	GenBank	Primer sequence	PCR product	PCR conditions
MMP-2	U07775.1	F: 5'-AGCTGCACCGTCACCAATCAT-3' R: 5'-CCTGCATCTGTGCAGCTGTTG-3'	668 bp	94°C 30s, 55°C 30s, 72°C 60s, 25 cycles
GADPH	NM_204305.1	F: 5'-GTGGAGAGATGGCAGAGGTG-3' R: 5'-AACAAGCTTGACGAAATGGT-3'	349 bp	95°C 30s, 52°C 15s, 72°C 30s, 27 cycles

1.5% agarose gel in 0.5x TBE buffer and stained with ethidium bromide. The gel was examined under UV light and the net intensities of individual bands were measured using the Scion Image for Windows. The ratio of net intensity of examined gene to GAPDH was used to represent the relative level of target gene expression. The average abundance of six repeats was used for statistical analysis.

Data were analyzed by ANOVA followed by a Tukey test. Significance of differences was considered at the level of $P<0.05$. Results are expressed as the mean ± SEM from 6 determinations.

Results

RT-PCR analysis showed the presence of MMP-2 mRNA in all examined compartments of the chicken ovary at two stages of the ovulation cycle, i.e. at 22 h and 3 h before ovulation. The amplification product consisted of a single band with the expected size of 668 bp for MMP-2 and 349 bp for GAPDH (Fig. 1).

The relative expression of MMP-2 mRNA within the small follicles ranged from 0.173 ± 0.043 in small yellow follicles (8-12 mm) to 0.675 ± 0.105 in white follicles (1-4 mm) at the stage of 22 h be-

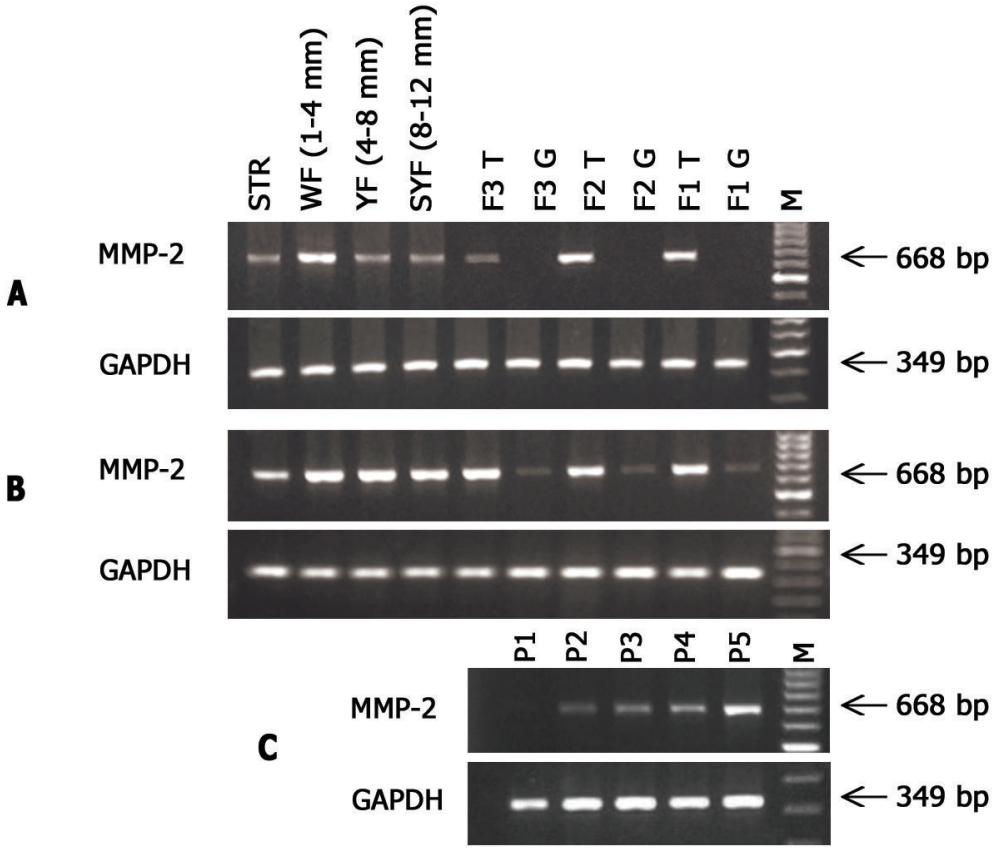

Fig. 1. Expression of MMP-2 mRNA in laying chicken ovary 22 h (**A** and **C**) and 3 h (**B**) before ovulation analyzed by RT-PCR. STR – stroma; WF – white follicles (1-4 mm); YF – yellowish follicles (4-8 mm); SYF – small yellow follicles (8-12 mm); F3-F1 – yellow preovulatory follicles: T – theca; G – granulosa; P1-P5 – postovulatory follicles; M – molecular weight marker (100 bp DNAladder).

fore ovulation and from 0.456 ± 0.098 in stroma with primordial follicles to 0.741 ± 0.054 in small yellow follicles 3 h before ovulation (Fig. 2). At the stage of 22 h before ovulation the expression of MMP-2 mRNA in small yellow follicles was significantly lower than in the group of white (1-4 mm) and yellowish (4-8 mm) follicles by 74.4%, and 67.8%, respectively. At the stage of 3 h before ovulation the relative level of MMP-2 mRNA in the stroma was significantly lower than in white follicles by 34% and small yellow follicles by 38.5% (Fig. 2). In the stroma and white and yellowish follicles there were no differences in MMP-2 mRNA expression between the examined stages, whereas in small yellow follicles expression was significantly higher 3 h than 22 h before ovulation (by 328%).

In the wall of preovulatory follicles F3-F1, the expression of MMP-2 mRNA was significantly lower in the granulosa layer than in the theca layer at both examined stages. In the theca layer of F3-F1 follicles the expression of MMP-2 ranged from 0.236 ± 0.049 to 0.539 ± 0.100 at the stage of 22 h before ovulation and was significantly higher in follicle F2 than in F3 (by 128%). At the stage of 3 h before ovulation the relative expression of MMP-2 was from 0.621 ± 0.120 to 0.715 ± 0.058 and did not differ among follicles (Fig. 3). In follicles F3 and F1 the MMP-2 expression was significantly higher at the stage of 3 h than 22 h before ovulation, by 177% and 77%, respectively. In the granulosa layer of F3-F1 follicles the relative expression of MMP-2 ranged from 0.006 ± 0.004 to 0.09 ± 0.037 22 h before ovulation and from 0.096 ± 0.044 to 0.106 ± 0.042 3 h before ovulation. There were no differences in MMP-2 expression among follicles and between examined stages (Fig. 3).

In the postovulatory follicles P1-P5 the expression of MMP-2 mRNA increased gradually from 0.092 ± 0.041 in P1 to 0.908 ± 0.077 in P5. A sharp, statistically significant increase (by 271%) was observed during the transition from P2 to P3 (Fig. 4).

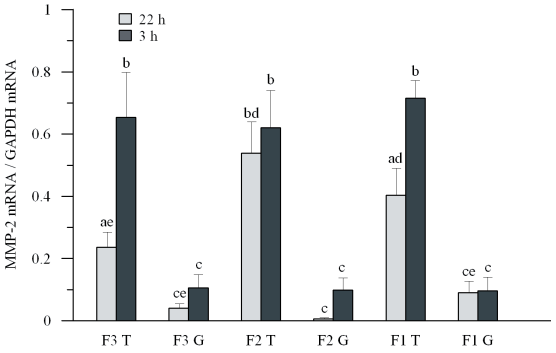

Fig. 3. Relative level of MMP-2 mRNA in the theca and granulosa layers of yellow preovulatory follicles of chicken 22 h and 3 h before ovulation determined by RT-PCR. Each value represents the mean ± SEM from 6 determinations of MMP-2 that were normalised with respect to GAPDH mRNA expression; values with different superscript letters differ significantly (P<0.05). F3-F1 – yellow preovulatory follicles: T – theca; G – granulosa

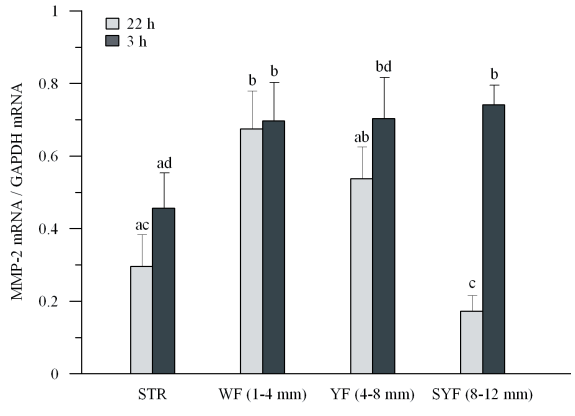

Fig. 2. Relative level of MMP-2 mRNA in the stroma, white, yellowish and small yellow follicles of chicken 22 h and 3 h before ovulation determined by RT-PCR. Each value represents the mean ± SEM from 6 determinations of MMP-2 that were normalised with respect to GAPDH mRNA expression; values with different superscript letters differ significantly (P<0.05). STR – stroma; WF – white follicles (1-4 mm); YF – yellowish follicles (4-8 mm); SYF – small yellow follicles (8-12 mm).

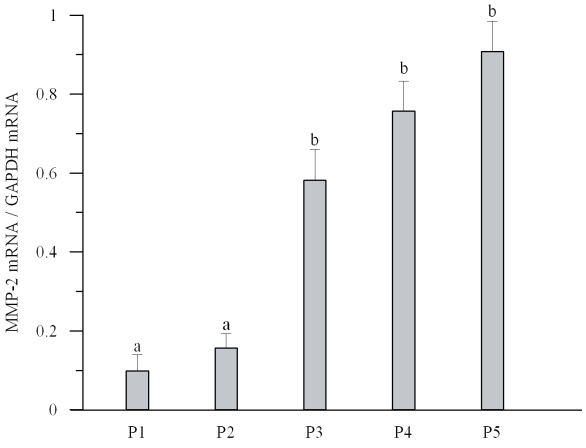

Fig. 4. Relative level of MMP-2 mRNA in postovulatory follicles (P1-P5) of chicken 22 h before ovulation determined by RT-PCR. Each value represents the mean ± SEM from 6 determinations of MMP-2 that were normalised with respect to GAPDH mRNA expression; values with different superscript letters differ significantly (P<0.05).

Discussion

This study showed the distribution of MMP-2 mRNA in different compartments of the chicken ovary, i.e. stroma, white follicles, yellowish follicles, small yellow follicles, three of the largest yellow preovulatory follicles (F3-F1) and postovulatory follicles (P1-P5). Thus, this finding demonstrates that MMP-2 mRNA is expressed in the follicular wall throughout the period of development until ovulation and subsequent regression of the follicle, and indicates the involvement of MMP-2 in regulation of these processes. The results, obtained for the first time in birds, are consistent with those previously reported in the mammalian ovary including the horse (SONG *et al*. 1999; RILEY *et al*. 2001; SESSIONS *et al*. 2009), mouse (HÄGGLUND *et al*. 1999; LIU *et al.* 1998, 2003), rat (LIU *et al.* 1999; CURRY *et al*. 2001), sheep (MURDOCH & McCORMICK 1992), cattle (SMITH *et al*. 1996), pig (BASINI *et al*. 2011) and human (DUNCAN *et al*. 1998).

The relatively higher expression of MMP-2 mRNA in the white follicles than in the ovarian stroma seems to be related to its participation in the process of atresia which is the most abundant in the group of white follicles. It is suggested that enhanced activity of proteolytic enzymes such as MMP-2 may facilitate the basement membrane breakdown characteristic of the later stages of atresia. HUET *et al.* (1998), for instance, have reported elevated activity of MMP-2 in ovine atretic follicles following hypophysectomy. In turn, the significantly elevated expression of MMP-2 in the small yellow follicles at the stage 3 h before ovulation may be related to the selection of these follicles to the preovulatory hierarchy in which follicles grow very fast and during several days reach a diameter of about 36 mm in the chicken. These results are at least partially consistent with those from studies on rodents. Namely, LIU *et al.* (1998) observed up-regulated expression level of MMP-2 mRNA during follicular development in the rat. Stimulation of follicular growth with equine chorionic gonadotropin (eCG) correlated with an enhanced expression of gelatinase A mRNA in the developing follicles. On the other hand, in the equine ovary RILEY *et al.* (2004) did not detect any significant changes in follicle MMP-2 secretion during the growth phase of the follicle. This difference may be due to species specificity and applied detection method.

A more interesting finding of the current study was the observation that at both stages of the ovarian cycle, MMP-2 mRNA expression in the theca layer of the large preovulatory follicles was very high, while in the granulosa layer it was very low, less detectable. This seems clear, because the theca layer is composed of collagenous fibres in large part, the target substrate for MMP-2 degradation. Otherwise, the theca layer has to be degraded in order to facilitate follicular rupture. Moreover, the breakdown of the basement membrane, demarcating the theca and granulosa layers, also requires degradation to release the oocyte during ovulation (ESPEY 1980). During the passage of the follicles from F3 to F1 position the expression of MMP-2 mRNA in both examined layers of the follicular wall did not change significantly, except for the lower expression in the theca layer of F3 than F2 follicles 22 h before ovulation. Compared between stages of the ovulatory cycle, increased MMP-2 mRNA expression was noted 3 h before ovulation in the theca layer of F3 and F1 follicles. Recently, MMP-2 expression was demonstrated in the theca and granulosa layer of porcine ovarian follicles (BASINI *et al*. 2011). In contrast to our results, these authors revealed that during follicular growth the expression of MMP-2 increases in the theca layer but decreases in the granulosa layer. A lack of spectacular changes in the expression of MMP-2 mRNA in the theca and granulosa layers of preovulatory follicles in the chicken may suggest that this MMP is not the only crucial factor involved in the process of ovulation in birds. On the other hand, it should be noted that in the current investigation the mRNA level was examined, and for ECM component degradation the active enzyme is necessary. MMP-2 activity is strictly controlled by tissue inhibitors of MMPs (TIMPs) (LAMBERT *et al*. 2004). To date the expression of TIMPs was detected only in the basal lamina of chicken preovulatory follicles (ASEM *et al*. 2000).

The growth and development of follicles until ovulation is accompanied by changes in steroid production and steroid receptor expression (YOSHIMURA & BAHR 1991; YOSHIMURA *et al*. 1993; HRABIA *et al*. 2008). It is well established in birds that secretion of estradiol decreases while progesteron increases during maturation of ovarian follicles (for a review see JOHNSON & WOODS 2007). Thus steroids, as key regulators of ovarian processes, may control MMP-2 expression and activity. In support of this hypothesis is the observation that changes in MMP-2 mRNA expression found in the present study correlate with changes in expression of estrogen receptor mRNA in the ovarian follicles (HRABIA *et al*. 2008). Moreover, HA *et al*. (2004) revealed that the expression of MMP-2 mRNA in the right Müllerian duct of the chicken embryo was decreased by administration of diethylstilbestrol, an estrogenic hormone.

The next interesting finding of our study was the gradual increase in MMP-2 mRNA expression in postovulatory follicles along with their progressing regression. These observations are in line with

the previous one, in which gelatinase A was found to be involved in the regression of the right Mullerian duct in female embryos of the chicken (HA *et al.* 2004). It was also shown that MMP-2 is expressed in the regressing corpus luteum in the mouse and rat ovary (LIU *et al*. 1999, 2003). Moreover, in the chicken postovulatory follicle, elevation in collagenase activity concurrent with time after ovulation was reported (TOJO *et al*. 1982). Regression of avian postovulatory follicles is accompanied by the disappearance of the basement membrane, lipid degeneration of granulosa cells and translocation of the theca and granulosa cells towards the follicle lumen. Our results strongly suggest that MMP-2, by degradation of ECM, participates in postovulatory follicle regression.

In conclusion, the results demonstrate the presence and differences in expression of MMP-2 mRNA in the compartments of the chicken ovary and indicate that MMP-2 may take part in extracellular matrix remodelling required for follicle growth, development and regression. To clarify the role of MMP-2 in the avian ovary, further experiments examining MMP-2 activity are needed.

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