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Matrix Metalloproteinases 2 and 9 and their Tissue Inhibitors in the Follicular Fluid of Patients with Polycystic Ovaries Undergoing *In Vitro* Fertilisation

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Abstract. *The present study was undertaken to investigate the levels of matrix metalloproteinase (MMP)-2, MMP-9 and their tissue inhibitors (TIMP-2 and TIMP-1, respectively) in the follicular fluid of 39 patients with polycystic ovary syndrome (PCOS) and compare them with the levels found in 56 age- and weight-matched normally ovulating women, all undergoing in vitro fertilisation (IVF) treatment. Significantly higher levels of MMP-2 and MMP-9 ($p=0.02$ and $p<0.001$, respectively) as well as TIMP-2 and TIMP-1 ($p=0.006$ and $p<0.001$, respectively) were found in the PCOS group compared to controls. Women who achieved pregnancy had higher TIMP-1 levels compared to the non-pregnant ones in the control group ($p=0.01$). In conclusion, women with PCOS exhibited significantly increased gelatinolytic activity compared with controls of similar age and body mass index, thus indicating a more intense extracellular matrix remodelling in this group of patients during IVF treatment due to multiple follicular development and cyst formation.*

During reproductive life, human ovarian tissue is subjected to a continuous and extensive remodelling process in relation to follicular growth, ovulation and atresia. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases that are involved in extracellular matrix (ECM) remodelling during ovarian follicular growth and ovulation. Their activity is regulated at different levels by complex mechanisms including specific interactions with tissue inhibitors of metalloproteinases (TIMPs) which represent a family of polypeptides that bind to MMPs and inhibit their activity. In particular, TIMP-1 regulates the activity of MMP-9 and TIMP-2 inhibits MMP-2 (1).

Despite the fact that the production of MMPs and TIMPs as well as their mechanisms of action in human ovaries are not fully understood yet, their presence in the follicular microenvironment is important for the subsequent follicular development. Deficient follicular growth and/or ovulation have been correlated with the presence of low levels of MMPs in follicular fluid (2). Gelatinases seem to have an important role in the stabilisation of the ECM, an important process at the initiation of pregnancy (3).

By definition, the polycystic ovary syndrome (PCOS) implies the presence of multiple cystic follicles together with many other morphologic changes in the ovaries, which results in a continuous and increased remodelling of the ovarian stroma. Increased serum concentrations of MMP-2 and MMP-9 have been reported in women with PCOS (4).

This study was based on the hypothesis that since tissue remodelling in polycystic ovaries is more intense than in normal ovaries, some differences in the activity of these parameters are expected between the two groups. Therefore, the study was designed to detect the levels of MMP-2, MMP-9 and their tissue inhibitors (TIMP-2 and TIMP-1, respectively) in the follicular fluid of PCOS patients undergoing controlled ovarian stimulation and compare them with the levels found in normally ovulating women under *in vitro* fertilisation (IVF) treatment.

Patients and Methods

Subjects and IVF treatment. The Institutional Review Board of Aretaieion Hospital approved the study and signed informed consent was obtained from all participants before recruitment. The

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present study involved 39 women with diagnosed PCOS, based on the evidence of any two of three features – hyperandrogenism, menstrual irregularity, and polycystic ovarian morphology on ultrasound examination, according to the Rotterdam consensus criteria (5) – and 56 age- and weight-matched non-PCOS control women enrolled for IVF. All of the control group had normal findings on pelvic ultrasound scan, regular periods and no clinical findings of hyperandrogenism on examination. None of these women, PCOS and controls, were on any medication for at least 3 months before the study. The body mass index (BMI) was calculated as body weight in kilograms divided by height in meters squared (kg/m^2).

In all patients, a long protocol, comprised of daily administration of GnRH analogue, was started beginning from the 21st day of the pre-treatment cycle (which was normalised with the administration of combined oral contraceptive) and initiation of recombinant FSH (beta-follitropin; Puregon; Organon, Oss, the Netherlands) in a daily dose of 75-300 IU (depending on the patient's characteristics) when down-regulation was confirmed (serum oestradiol <50 pg/ml). Follicular monitoring was accomplished by serum oestradiol measurements (using the Abbott Architect i-1000 SR autoanalyzer, Abbott Laboratories, North Chicago, IL, USA; sensitivity 10 pg/ml, intraassay CV 5.5%, interassay CV 6.7%) and serial ultrasonographic examinations. Human chorionic gonadotropin (hCG; 10,000 IU) was administered when 3 or more follicles 18 mm in diameter were detected on transvaginal ultrasound. Oocyte pick-up was scheduled 35-36 hours post-hCG and was performed transvaginally under ultrasound. Fertilisation was performed with the respective husband's spermatozoa, and all embryo transfers (ETs) were performed on day 3 with a Wallace catheter under ultrasound guidance. Pregnancy was defined as the presence of a gestational sac containing a foetal pole under ultrasound examination.

Sample collection and measurements. Blood-free follicular fluids (FF) were collected during oocyte pick-up from follicles reaching about 18 to 20 mm in diameter after removing the oocytes. In each patient, FF samples were pooled and a volume of about 5 ml (after centrifugation at 600 xg for 10 minutes) was stored at –80˚C until analysis.

The concentrations of MMP-2, MMP-9, TIMP-2 and TIMP-1 were determined in duplicate using the respective RayBio® Human MMP-2, MMP-9, TIMP-2 and TIMP-1 ELISA kits (RayBiotech Inc, Norcross, GA, USA) designed to measure their pro and active forms. The minimal detectable concentration was 80 pg/ml for MMP-2, 10 pg/ml for MMP-9 and TIMP-2 and 40 pg/ml for TIMP-1. The intra-assay and inter-assay CV for all enzymes were <10% and <12%, respectively.

Statistical analysis. Statistical analysis of the data was performed using Student's *t*-test, Fisher exact test and Mann-Whitney test. Data are expressed as the mean±standard deviation (SD). The cut-off value for significance was set at 0.05.

Results

In this study, 39 women with PCOS were analysed and compared with 56 normal ovulatory women who served as controls. Patient characteristics can be seen in Table I. The two groups did not differ in age (p=0.064), in the number of embryos transferred (p=0.08), nor in pregnancy rates (p=0.07). Moreover, the two groups did not differ in BMI, duration of infertility, number of previous IVF-embryo transfer (IVF-ET) cycles, nor the total amount of gonadotrophins administered (data not shown). In contrast, in the PCOS group there were significantly more oocytes retrieved (p<0.001) as well as the number of fertilised oocytes (p<0.001) compared to the control group.

Activity of both MMPs and TIMPs varied greatly between patients. MMP-2 and MMP-9 levels were significantly higher (p=0.02 and p<0.001, respectively) in the PCOS group compared to the control group. The same was true for their inhibitors, TIMP-2 and TIMP-1 (p=0.006 and p<0.001, respectively). No statistically significant differences were seen when the levels of any of the parameters under investigation were compared in women who became pregnant. The only exception was that higher TIMP-1 levels were recorded in women who achieved pregnancy (1,397.5±366.0 ng/ml) *versus* those who were not pregnant (1,099.0±515.5 ng/ml) by the end of the IVF cycle in the control group (p=0.01). Furthermore, although significant correlations were observed among the number of oocytes retrieved, number of embryos obtained, oestrogen levels and MMP-2, MMP-9 and TIMP-1 when all women were taken into consideration, these correlations were not consistent when the two groups were investigated separately (Table II).

Discussion

In the present study, it was demonstrated that PCOS patients showed higher follicular fluid levels of MMP-2 and MMP-9 together with their inhibitors, TIMP-2 and TIMP-1, respectively, compared to the levels found in normally ovulating women undergoing IVF treatment.

During reproductive life, ovarian tissue is subjected every month to profound and successive changes in relation to the follicular development and the breakdown of the follicular wall at ovulation, phenomenon more intense during ovarian hyperstimulation. An important role in all these changes has been attributed to the MMP-TIMP system (1). At ovulation, the increase in ovarian collagenolysis has been associated with an increase in MMPs expression and activity (6). D'Ascenzo *et al*. (2) reported for the first time that MMP-2 and MMP-9 levels were much lower in FF of IVF patients compared with that of normally ovulating women. They presumed an important role for the MMP-2 in follicular development and for the MMP-9 in follicular breakdown. In addition, TIMP-2 levels were similar in both groups, while TIMP-1 expression was higher in the IVF patients compared to the controls. In contrast, no statistically significant differences were observed in the MMP-2 and MMP-9 FF levels between natural cycle women and different stimulation protocols (7).

Table I. *Characteristics of study patients.*

Characteristic	PCOS	Control	*p*-Value
No. of cases	39	56	–
Age (years)	32.3±4.0	33.9±4.3	0.064
BMI (kg/m^2)	22.4±0.2	22.5±0.2	NS
No. of oocytes	10.0±2.2	4.9±2.5	<0.001
No. of fertilised oocytes	7.2±2.7	3.4±1.9	<0.001
No. of embryos for ET	2.9±0.9	2.6±1.1	0.08
No. of pregnancies	16	14	0.07
MMP-2 (pg/ml)	741.9±726.4	544.7±600.3	0.02
MMP-9 (pg/ml)	4,734.3±1,899.8	2,991.9±1,384.9	<0.001
TIMP-1 (ng/ml)	1,639.8±267.4	1,173.6±496.8	<0.001
TIMP-2 (ng/ml)	641.5±98.7	550.4±144.4	0.006

PCOS, Polycystic ovary syndrome; BMI, body mass index; ET, embryo transfer; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Values for MMPs and TIMPs are means±SD. NS, not significant; Nr, number.

Table II. *Correlations among study parameters in patients (values correspond to r coefficient of correlation).*

Patients	Oocytes	Embryos	E_2
All women			
MMP-2	0.29 (p=0.003)	0.28 (p=0.005)	0.22 (p=0.02)
MMP-9	0.43 (p<0.0001)	0.47 (p<0.0001)	0.47 (p<0.0001)
TIMP-1	0.55 (p<0.0001)	0.55 (p<0.0001)	0.45 (p<0.0001)
TIMP-2	0.19 (p=NS)	0.14 (p=NS)	0.17 (p=NS)
PCOS group			
MMP-2	0.11 (p=NS)	0.09 (p=NS)	0.04 (p=NS)
MMP-9	–0.05 (p=NS)	0.32 (p=0.04)	0.19 (p=NS)
TIMP-1	0.06 (p=NS)	0.18 (p=NS)	0.13 (p=NS)
TIMP-2	–0.18 (p=NS)	–0.17 (p=NS)	–0.19 (p=NS)
Control group			
MMP-2	0.28 (p=0.04)	0.21 (p=NS)	0.13 (p=NS)
MMP-9	0.32 (p=0.01)	0.13 (p=NS)	0.27 (p=0.04)
TIMP-1	0.43 (p=0.0009)	0.43 (p=0.001)	0.09 (p=NS)
TIMP-2	0.06 (p=NS)	–0.02 (p=NS)	0.03 (p=NS)

E_2, Estradiol; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; PCOS, polycystic ovary syndrome; NS, not significant.

In patients with PCOS, MMP-2 and MMP-9 have been associated with inappropriate follicular development (8). Lahav-Baratz *et al*. (9) found similar activity of MMP-2 and MMP-9 in the FF of leading follicles in normally ovulating and women with PCOS. In contrast (and in concordance with the results of the present study), elevated FF levels of MMP-2 and MMP-9 were reported in women with PCOS compared to normally ovulating women (10); this may be related to an increased ovarian ECM remodelling with multiple cyst formation. Interestingly, when only FF from leading follicles was examined, the levels found were similar between the 2 groups (9). These differences may be explained by the hormonal environment within a specific follicle and by possible intrinsic differences between different-sized follicles (11). In order to overcome possible variations between different-sized follicles and to obtain a more representative sample for each patient pooled FFs were used in the present study.

At ovulation, an increase in MMP expression and activity was noted which was attenuated by an increase in TIMP production (6). An interesting observation concerning the decrease in TIMP-1 expression in patients with PCOS was regarded as a compensatory process to overcome the thick ovarian capsule (9). In their study, Riley *et al*. (12) observed high levels of TIMP that remained unchanged during follicular development. TIMP-2 activity increased in response to the gonadotropin surge, supporting the hypothesis that its expression may help regulate follicle rupture and/or the transition to *corpus luteum* (8). In the present study, TIMP levels were closely related to the changes in MMPs as previously reported (13). High TIMP levels are needed in order to inhibit MMP activity (13).

Significant correlations were detected between all the parameters under investigation (with the exception of TIMP-2) and the number of oocytes, embryos and oestrogen levels when all women were taken into consideration. Nevertheless, analysing these correlations separately in the two study groups, the above correlations were not confirmed, which is probably explained by the small number of participants in each group.

Pregnancy represents a period of major changes in the uterus, so variations in MMP concentrations could be expected. Nevertheless, studies revealed that MMP-2 levels in serum (14) or in FF (15) were not different between pregnant and non-pregnant women, suggesting that evaluation of this MMP may not be useful for the management of infertile women after IVF-ET. In contrast, higher MMP-9 levels in FF were found in the pregnant group compared to the non-pregnant group in IVF cycles (15). In the present study, no differences in the FF MMP-2 and MMP-9 levels were observed in relation to pregnancy in either group and this observation is in agreement with a previous study (16) and with the hypothesis presented by Stamouli *et al*. (3) stating that the rescue of the *corpus luteum* in early pregnancy involves the maintenance of cellular function through the stabilisation of the ECM. It seems that, at least at the initiation of pregnancy, the modifications that are required in the ECM do not involve great variations in MMP levels. Although the small number of participants included is a limitation in this study, the presented results do not support the consideration of either of the MMPs studied as being predictors for the establishment of pregnancy. In contrast, the significantly increased TIMP-1 levels in the control women who became pregnant was an unexpected finding that could be explained by the already higher levels due to the IVF

treatment as previously demonstrated (2) and/or by the decreased levels in PCOS women as a result of the process to overcome the thick ovarian capsule (9). Lastly, the observed higher TIMP-1 levels may have a compensatory effect to inhibit the MMP-9 activity, which was previously proposed as a marker for successful outcome in IVF (15). In the population studied in the present study, MMP-9 levels did not differ between the groups, probably due to the small number of participants included.

In summary, the findings of the present study underline a marked difference in MMP-2, MMP-9 and their inhibitors TIMP-2 and 1, respectively, between PCOS and normal ovulatory women under IVF treatment. Moreover, it seems that MMP and TIMP thresholds are elevated in PCOS patients irrespective of number of oocytes, embryos or IVF outcome, influenced by mechanisms not fully understood yet. The fact that all parameters under consideration displayed considerable variations in their levels both between the two groups of the study, as well as among the patients of the same group, probably indicates that levels may be regulated by the hormonal milieu within each patient. Further studies focusing on potential modulators of the MMP and TIMP levels in the FF of PCOS patients could be useful for a better understanding of the mechanisms involved in the profound ovarian reconstruction and follicular development during controlled ovarian hyperstimulation.

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