DOI:10.2478/jbcr-2019-0015

Original Articles

LEVELS OF MMP2, TIMP1 AND TIMP2 IN FOLLICULAR FLUIDS IN WOMEN UNDERGOING IN VITRO FERTILIZATION AND THEIR RELATIONSHIP TO OOCYTE QUALITY

Nina P. Ayvazova[1], Lyubomira O. Ilieva[2], Emiliana I. Konova1,[2], Milena A. Atanasova[3]

[1] Medical Center Clinical Institute for Reproductive Medicine – Pleven, Bulgaria
[2] Department of Clinical Immunology, Allergology and Clinical Laboratory, Medical University – Pleven, Bulgaria
[3]Division of Biology, Medical University – Pleven, Bulgaria

Summary

Recently, the important role of matrix metalloproteinases (MMPs) has been identified in follicular development and subsequent ovulation. Although the role of MMP in ovarian tissue remodeling during folliculogenesis has been well studied, the relationship between matrix protease activity and their inhibitors – Tisue inhibitors of matrix metalloproteinases (TIMP) and aging of the oocytes is still unclear. The present study aimed to establish the probable relationship between the expression levels of MMP-2 and TMP-1 and TIMP-2 in follicular fluid with the degree of oocyte maturity and quality. Follicular fluids from 20 women collected on the day of follicular puncture were tested for the presence of MMP-2, TIMP-1, and TIMP-2 using enzyme-linked immunosorbent assay (ELISA). The oocytes obtained were described in terms of maturity, morphology, and fertilization, as well as the embryo's quality and rate of development. MMP-2 was significantly higher in follicular aspirates in the first prophase of meiosis – germinal vesicle (GV), compared to aspirates with first metaphase (MI) ($p=0.011$) and second metaphase (MII) of mature oocytes ($p=0.010$). The MMP-2/TIMP-1 ratio was significantly higher for GV compared to M1 ($p=0.011$), M2 ($p=0.006$) and atretic oocytes ($p=0.032$); ($F(3, 71)=2.909$, $p=0.040$). Based on our results, we can conclude that MMP-2 concentration in follicular fluids during the IVF / ICSI procedure had a significant relationship to oocyte maturation levels. It was significantly higher in the case of immature oocytes. On the other hand, oocytes with normal morphology were associated with a significantly higher MMP-2 concentration in follicular fluids.

Keywords: MMP-2, TIMP-1, TIMP-2, oocyte quality, follicular fluid

Corresponding Author:
Nina P. Ayvazova,
Medical University Pleven, Biology Division
1, Kliment Ohridski Str.
Pleven, 5800
Bulgaria
e-mail: ayvazovanina@abv.bg

Received: September 05, 2019
Revision received: November 18, 2019
Accepted: December 01, 2019

Introduction

The primary function of the human ovary is to produce oocytes suitable for fertilization. Follicular development, rupture of the follicular wall, corpus luteum formation, and luteolysis require extensive remodeling of tissues and extracellular matrix (ECM), cell migration, and rapid angiogenesis. Complexes of ECM components are detected deposited between the granulosa cells, thus polarizing and maintaining the functional specialization of the granulosa cell layer and the regulated entry of proteins into the intra-follicular

environment. The composition and integrity of the ECM regulate cell shape, communication between them, steroidogenesis [1, 2]. MMPs are a family of zinc endopeptidases capable of degrading all the components of the ECM and are subdivided into subgroups depending on the specificity of the substrates [3, 4]. Regulation of MMP activity occurs at the level of gene expression, protein secretion, zymogenic activation, and inactivation of active MMPs by one or more inhibitors. Alfa-2 microglobulin and tissue inhibitors of metalloproteinases (TIMPs) mainly inhibit MMPs. In mammals, four types of TIMP proteins (TIPM-1 to 4) have been identified, with the first two exhibiting inhibitory activity to the active forms of all currently known MMPs [5]. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) belong to the subgroup of gelatinases, and their activity is specifically inhibited by TIMPs. Tissue inhibitor of metalloproteinases-1 (TIMP-1) exhibits a higher affinity for MMP-9, whereas tissue inhibitor of metalloproteinases-2 (TIMP-2) has a higher affinity for MMP-2 [6]. MMP systems are abundantly represented at the sites of the active reorganization of the ECM, with a consistent expression of MMP and TIMP in the ovary, especially in forming corpus luteum. MMP-TIMP system is involved in the proteolytic network of follicular development and rupture of the follicle wall with successful ovulation. In vitro studies with animal models have revealed that secretion of MMP-9, TIMP-1, and TIMP-2 is associated with follicular quality [7]. Several studies have established the relationship of MMP-2 to oocyte maturation and fertilization levels [8-10]. The imbalance in MMP / TIMP secretion is associated with conditions of impaired ovarian function, such as polycystic ovarian syndrome [11, 12]. Impaired MMP / TIMP secretion, along with increased perifollicular infiltration of leukocytes, have been reported in follicular atresia [10, 11]. Although the role of MMP in ovarian tissue remodeling during folliculogenesis has been well studied, the relationship between matrix protease activity and their inhibitors and oocyte aging is still unclear [3]. Oocytes collected during follicular puncture after controlled ovarian hyperstimulation (COXC) have a different degree of maturity and quality. It was found that between 5-20% of the removed oocytes are immature and have a low fertility rate [13]. Finding an accurate marker for the degree of oocyte's maturity is essential for successful assisted reproduction. The present study aimed to establish the probable relationship between the expression levels of MMP-2 and TMP-1 and TIMP-2 in follicular fluid with the degree of oocyte maturity and quality. The oocyte quality assessment was performed based on the following morphological features: the degree of cumulus complex expansion (COC), oocyte maturity, zona pellucida (ZP) defects, perivitelline space (PVS) defects, oocyte cytoplasm dysmorphism, and polar body (PB) morphology.

Materials and Methods

Patients

The study included 20 women undergoing controlled ovarian hyperstimulation and subsequent in vitro fertilization. To exclude the influence of the male factor, only pairs with partner's semen parameters meeting the following requirements were selected: total concentration of motile sperm> 10x106 per milliliter; DNA Fragmentation Index (DFI) <20%.

Collection of follicular aspirate

On the day of follicular puncture (36 hours after triggering the ovulation with human chorionic gonadotrophin), all follicles sized >14 millimeters were aspirated. The aspirates were collected separately to provided traceability between the collected oocyte and the corresponding follicular fluid. After collecting the oocytes, the aspirates were centrifuged at 300 g for 10 minutes at 4°C, distributed into two tubes, and stored at - 70°C until assay.

The aspirated oocytes were evaluated in terms of the degree of maturity and morphological features. For each oocyte, the results from fertilization, development, and quality of the pre-implantation embryos obtained, as well as the outcome of the procedure, were described.

A total of 225 clear follicular aspirates were examined, and the concentrations of MMP2, TIMP1, and TIMP2 were determined for each aspirate. The obtained results were grouped according to the parameters compared, namely: a degree of COC expansion, oocyte maturity, defects in ZP, PVS, PB, and cytoplasmic

abnormalities. Similarly, the data from the resulting fertilization and embryo quality on days three and five were also processed. For day three, the main criteria for the embryo assessment were the number of blastomeres and the percentage of fragmentations. The criteria of Gardner et Schoolcraft (1999) [14] were used to evaluate blastocysts on day five.

Enzyme-linked immunosorbent assay (ELISA) for MMP and TIMP assessment

The concentration of MMP-2 and TIMP-1 and TIMP-2 in follicular fluids was determined by a commercially available kit (R & D Systems), according to the manufacturer's instructions. Each sample was tested in duplicate, and the average value was used to calculate the concentration. The concentration of MMP-2, TIMP-1, and TIMP-2 was expressed in nanograms per milliliter (ng/ml).

Statistical analysis

The obtained results were processed and analyzed with Statistical Package for Social Science - SPSS (version 19.0, Chicago, IL, USA). The data for each of the tested indicators were presented as mean ± SD. We used Student's t-test and one-way analysis of variance (ANOVA) to compare the individual groups. Values of $p < 0.05$ were considered statistically significant. A Pearson test was used to determine the correlations between the variables examined.

Results

Comparison of the concentrations of MMP-2, TIMP-1, and TIMP2 in follicular fluids with different degrees of COC expansion did not show any statistically significant dependencies and trends. The following significant differences in MMP-2 concentration and oocyte maturity were found in the analysis: MMP-2 was significantly higher in follicular aspirates, in which were found oocytes in the first prophase of meiosis (GV – germinal vesicle) compared to aspirates with first metaphase – MI, intermediate maturity oocytes (p=0.011) and second metaphase – MII (mature oocytes) (p=0.010) (Figure 1). Comparison of MMP-2 concentrations in follicular fluids with GV and atretic oocytes showed the same trend, even though not statistically significant (p=0.054);(F(3, 71)=2.60, p=0.059). Concerning the concentration of TIMP-1, there was a tendency for lower expression in GV aspirates compared to MI (p=0.054) and a significantly lower concentration of TIMP-1 in GV aspirates, as compared to those with atretic oocytes (p=0.027); (F(3, 71)=0.44, p=0.729).

When comparing the MMP-2/TIMP-1 ratio (Figure 2), we found that it was significantly higher for GV compared to M1 (p=0.011), M2

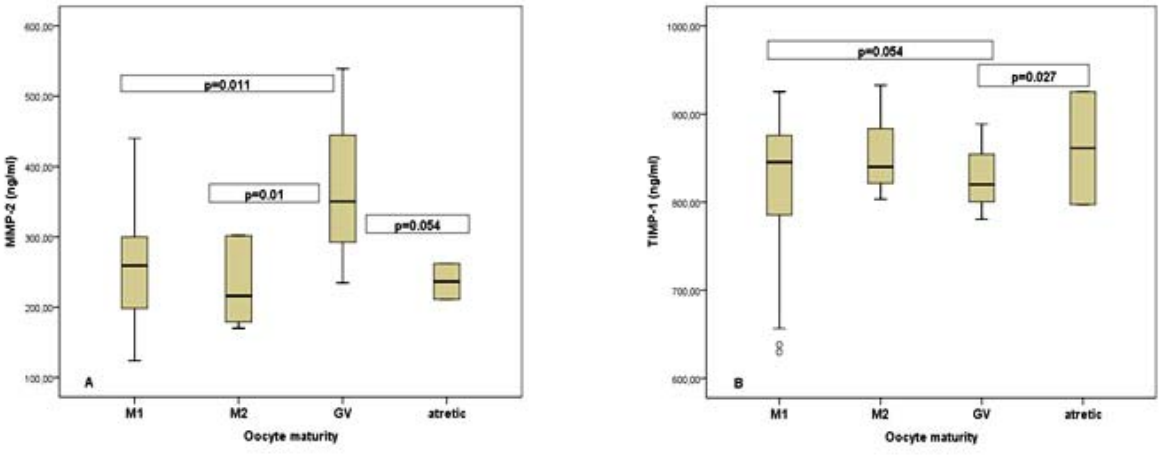

Figure 1. Dependancy of MMP-2 and TIMP-1 concentrations and oocytes maturity
The concentration of MMP2 was significantly higher in follicular fluids with immature oocytes (GV) compared to mature oocytes (M2). Comparing MMP-2 concentrations in follicular fluids with GV and atretic oocytes showed the same trend, even though not statistically significant (A). Concerning the concentration of TIMP-1, there was a tendency for lower expression in GV aspirates compared to MI and a significantly lower concentration of TIMP-1 in GV aspirates as compared to those with atretic oocytes (B).

(p=0.006) and atretic oocytes (p=0.032); (F(3, 71)=2.909, p=0.040). For TIMP-2, we did not find any differences for this criterion.

When examining the defects in the ZP and the PVS size, there were no statistically significant differences between the compared groups, but

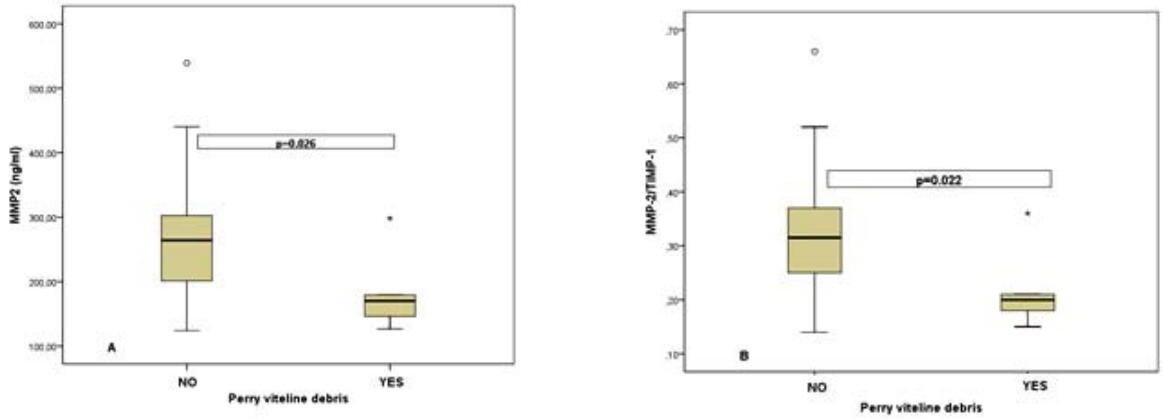

Figure 2. Correlation between the presence of perry vitelline debris and the concentration of MMP-2, and the TIMP-2/TIMP-1 ratio.
A significantly higher concentration of MMP-2 was found in follicular fluids with oocytes without fragments in PVS (A). The same dependence was also observed when comparing the ratio of MMP2 / TIMP1 (B).

we found a negative correlation between the concentration of MMP2 and the size of PVS (r=-0.253; p=0.030). A significantly higher concentration of MMP-2 was found in follicular fluids with oocytes without fragments in PVS (t(69)=2.16; p=0.026). The same dependence was also observed when comparing the ratio of MMP-2 / TIMP1 (t(69)=2.32; p=0.022)

A significantly higher concentration of MMP-2 was observed when oocytes were with a centrally granulated cytoplasm compared to

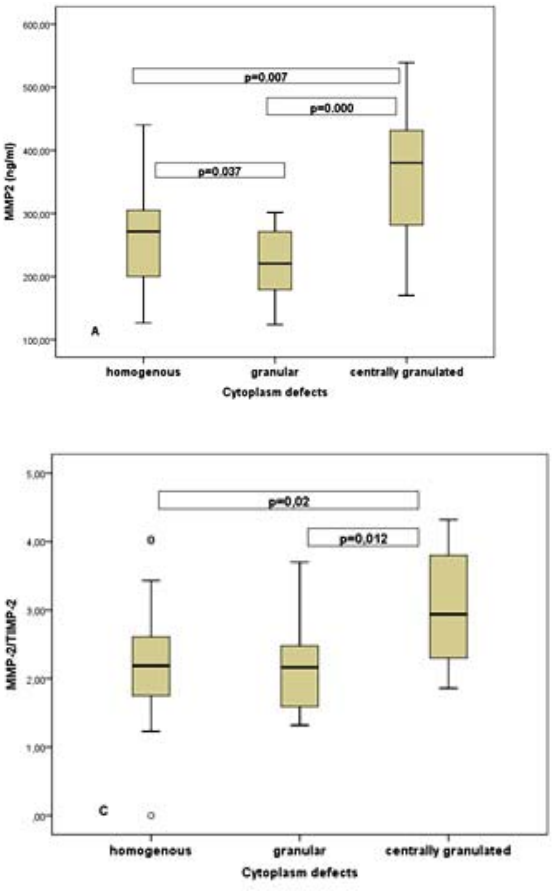

Figure 3. Correlation between oocyte's cytoplasmic morphology and the concentration of MMP-2 and its ratio with TIMP-1 and 2.
A significantly higher concentration of MMP-2 was observed when oocytes were with a centrally granulated cytoplasm compared to those with homogenous and granular cytoplasm. There was also a difference in the concentration of MMP-2 between the groups of oocytes with homogenous and granular cytoplasm, as in the group of oocytes with a homogeneous cytoplasm, the concentration of MMP2 was significantly higher (A). MMP-2/TIMP1 and MMP2/TIPM2 ratios were considerably higher in cases with centrally granulated cytoplasm oocytes compared to granular and homogenous cytoplasm (B - C).

those with homogenous (p=0.007) and granular cytoplasm (p=0.000) (Figure 3). There was also a difference in the concentration of MMP-2 between the groups of oocytes with homogenous and granular cytoplasm. In the group of oocytes with a homogeneous cytoplasm, the concentration of MMP2 was significantly higher (p=0.037); (F(2,68)=7.33, p=0.001). The concentration of TIMP-1 and TIMP-2 the three groups did not differ significantly, but the relations described above were observed again when comparing the ratios of MMP-2/TIMP-1 and MMP-2/TIMP-2 (p=0.011, p=0.000, p=0.012; F(2, 68)=8.13, p=0.001 and p=0.02, p=0.012; F(2, 68)=3.80'p=0.027)

Regarding the presence of cytoplasmic inclusions and vacuoles, there was no statistically significant difference in the concentration of MMP-2, TIMP-1, and TIMP-2, and their ratios (Figure 4). The MMP-2 concentration was higher in follicular fluid with normal polar body oocytes (t(73)=2.64, p=0.027), as compared to the group of oocytes with fragmented PB. We also established a negative correlation between MMP-2 concentration and PB defects (r=-0.302; p=0.009). The ratios of MMP-2/TIMP1-(t(73)=2.52, p=0.010) and MMP-2 /TIMP-2 (t(73)=2.14, p=0.01) were also significantly higher in the normal PB group.

No significant differences in MMP-2 and TIMP2 concentrations relative to embryo fertilization and development on day 3 and day 5 were observed, but a positive correlation between TIMP-1 concentration and oocyte fertilization was found (r=0.315; p=0.011).

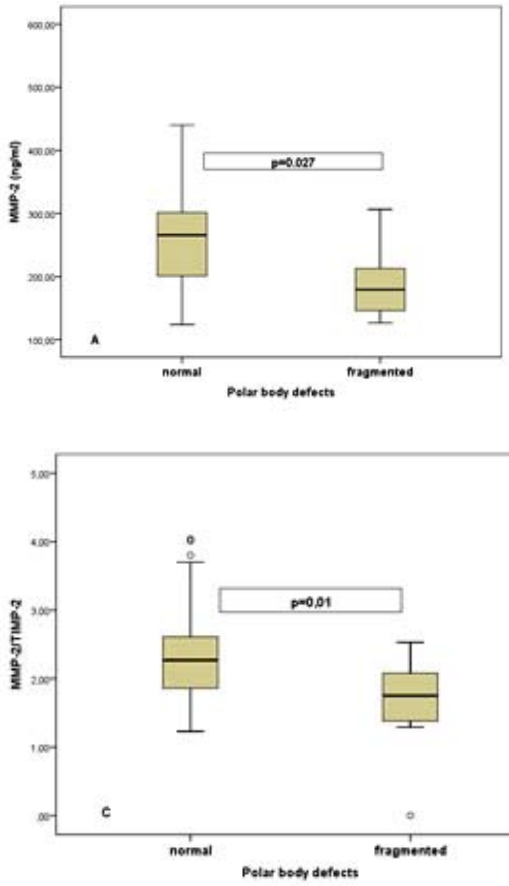

Figure 4. Correlation between Polar body (PB) defects and the concentration of MMP-2 and its ratio with TIMP-1 and 2.
The concentration of MMP2 was higher in follicular fluid with healthy polar body oocytes compared to the group of oocytes with fragmented PB (A). The ratios of MMP2 / TIMP1 (B) and MMP2 / TIMP2 (C) are also significantly higher in the healthy PB group.

Regarding the outcome of the procedure, a higher concentration of MMP-2 was observed in ovarian follicular fluids in cases where no pregnancy was achieved after embryo transfer (t(39)=0.407, p=0.041), as compared to those with positive pregnancy tests (Figure 5).

Discussion

Achievement of pregnancy in assisted reproduction depends on a variety of

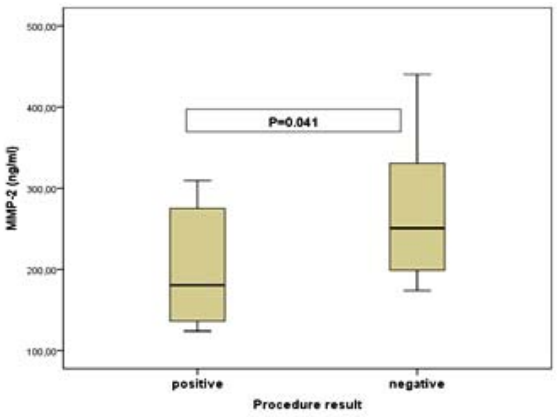

Figure 5. The concentration of MMP-2 and the outcome of the assisted reproduction procedure.
A higher concentration of MMP-2 was observed in ovarian follicular fluids, in cases where no pregnancy was achieved after embryo transfer, compared to cases with a positive pregnancy test.

physiological conditions, including oocyte maturity, successful fertilization, and embryonic development to the blastocyst stage [15]. At this point in the IVF / ICSI procedure, the main predictor of oocyte maturation depends mainly on the size of the leading follicles (>17 mm in diameter), combined with serum estradiol levels. However, there is still no accurate marker predictive of the degree of maturity of the oocytes. Recently, the critical role of MMPs in follicular development and subsequent ovulation has been identified [16-18]. Animal studies have shown the regulatory role of MMP-2 in ovulation and formation of the corpus luteum [19]. Yang et al. (2015) [8] established a correlation between MMP-2 activity and the degree of maturity of the oocytes. In the present study, the data indicated a similar relationship, but we found a markedly increased concentration of MMP-2 in follicles containing immature GV oocytes, as compared to MII. Matrix metalloproteinase-2 is thought to have an active role in the early development of follicles [16]. A rat study revealed a high immunoreactivity of MMP-2 in granulosa cells, and surface epithelium was found in follicles in the early stages of their development [20]. In humans, increased expression of MMP-2 mRNA was also found in cells surrounding small developing follicles, which confirmed the role of the enzyme in early follicular development [11]. Considering the wide variety of substrates and biological pathways that are affected by MMPs, their enzymatic activity is completely regulated at the level of gene expression, protein secretion, and zymogenic activation and is specifically inhibited by TIMPs. Increased TIMP expression was detected around the time of onset of ovulation [21], while other studies found increased MMP expression and decreased TIMP [22]. In our study, we did not establish a relationship between TIMP-1 and TIMP-2 concentration and oocyte maturity, which confirms the data from other similar studies [8]. The quality and potential for the development of the embryos obtained during assisted reproduction depend to a great extent on the degree of nuclear and cytoplasmic maturity of the oocytes. Usually, after controlled ovarian hyperstimulation, along with the normally ovulating follicles, oocytes are collected that would never reach ovulation under physiological conditions. The presence of various dysmorphisms in oocytes is associated with reduced fertility potential and poor quality of embryos. Dysmorphisms are conventionally divided into two groups: i.e., cytoplasmic (inclusions, refractile bodies, central granulation, vacuoles, smooth endoplasmic reticulum aggregates) and extracytoplasmic (PB morphology, PVS size and granulation, ZP defects and oocyte-shape abnormalities). The presence of fragmented PB and increased PVS size, as well as fragments in PVS, are indicators of oocyte aging [23]. Our results showed a significant increase of MMP-2 concentrations in follicular aspirates containing oocytes with normal morphology of PB and PVS. This finding supports the theory of the leading regulatory role of MMP-2 in the pre-ovulatory development of the follicles. It could be speculated that the increased secretion of MMP-2 in follicular fluid is an indicative marker for the quality of the corresponding oocytes.

On the other hand, there is a correlation between increased enzyme concentration and the presence of centrally granulated cytoplasm, dysmorphism that is thought to arise in the early stages of oocyte maturation and has been associated with reduced fertility, as well as an increased risk of aneuploidy [24]. The established significantly higher MMP-2 concentration in the cases of absence of embryo implantation indicates that the production of

mature and qualitative oocytes from the ovary is a result of a delicate balance of expression and enzymatic activity of MMP-2, as well as the involvement of other factors. In this case, the increased concentration of MMP-2 refers to the functional immaturity of the oocytes.

Regarding the concentration of TIMP-1 and TIMP-2, no significant differences were found for any of the tested groups concerning the oocytes morphology. In this study, we did not establish a relationship between MMP-2 and TIMP-2 concentrations and the fertilization index and the quality of the embryos obtained. TIPMs expression in the ovary reaches its maximum around the time of ovulation [21]. In addition to inhibiting MMP activity, TIMPs play a role in regulating cell growth and apoptosis processes by binding to specific cellular receptors [25]. At autocrine and paracrine levels, they also participate in the regulation of cell proliferation, differentiation, neovascularization, and steroidogenesis in growing follicles [16]. The regulation of steroid synthesis by TIMP-1 occurs at significantly lower concentrations than those required for inhibition of MMPs [24, 26]. These facts could explain, to a certain extent, the positive correlation we found between the concentration of TIMP1 and oocyte fertilization.

Conclusions

In conclusion, we could summarize that the relationship between MMP-2 and oocyte maturation was confirmed in the present study, with a likelihood that the enzyme concentration also affects the quality of the oocytes. Further studies are needed to establish how the levels of enzyme expression and activity of MMPs and their inhibitors influence follicular development and how this reflects the ovarian response during controlled ovarian hyperstimulation.

Acknowledgements

This work was supported by project D2/2017 of Medical University – Pleven.

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