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Hyaluronidase Inhibits the Proliferation and the Viability of Cumulus Cells Through the Inhibition of Midkine

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ABSTRACT

OBJECTIVE: Hyaluronidases (HYAse) break down hyaluronic acid, which is found in high levels in the cumulus-oocyte complex during oocyte preparation for IVF. Using HYAse, we aimed to investigate whether a growth factor, midkine (MK), has a role in the proliferation and viability of cumulus cells (CCs) during the denudation process.

STUDY DESIGN: A prospective, randomized study was done with 90 females aged 21-40 undergoing ICSI from September 2017 to September 2018. CCs were cultured via a mechanical application from male factor cases at the IVF clinic of a private hospital. HYAses in concentrations of 0.1 IU/mL (The lowest concentration), 1 IU/mL, and 10 IU/mL (The highest concentration) were applied to CCs. Every 48 hours of 24 hours, cell proliferation and apoptosis indices (Flow cytometry) with MK levels (ELISA) and the ultrastructure of cumulus cells (TEM) at the 48th were evaluated. A one-way ANOVA test was used, and $p<0.05$ was considered statistically significant.

RESULTS: All concentrations decreased the cumulus cell numbers for 48 hours. The highest decrease in cell number was detected at the highest concentration at the 48th h. In concordance with this result, the highest increase in apoptotic and dead cell rates with the lowest viable cell rate was detected at the highest concentration at the 48th h. The highest concentration led to the highest decrease in MK levels at the 48th h.

CONCLUSIONS: HYAse inhibits the proliferation of CCs by inhibiting MK in a time and concentration-dependent manner.

Keywords: Apoptosis; Hyaluronidase; In vitro fertilization; Midkine; Oocyte quality

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Introduction

In clinical practice, cumulus cells (CCs) are usually mechanically or enzymatically (Frequently used hyaluronidase) removed from cumulus-oocyte complexes (COCs). Hyaluronidase (HYAse) is generally used to remove cumulus cells from oocytes before oocyte cryopreservation at in vitro fertilization (IVF). An enzyme HYAse hydrolyzes hyaluronan (hyaluronic acid, hyaluronate), which is a polysaccharide consisting of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid (1). Highly hydrated hyaluronan composes a viscoelastic matrix in the extracellular space of COCs. HYAse application results in the degradation of the hyaluronan-based matrix surrounding COCs and splits the cumulus cells from oocytes (2).

Studies proved that handling fully denuded cumulus-free oocytes decreases IVF ability compared to cumulus-surrounded oocytes, which have reduced cumulus layers (3-5). Consequently, some layers of cumulus cells, such as 3 or 4, were preserved to maintain oocyte viability and quality (6). It has been shown that the oocyte and surrounding CCs have an interdependent relationship in the early stage of folliculogenesis (7). CCs possess important roles in oocyte development through the secretion of growth hormones and ovarian steroid hormones (8). However, atresia is triggered by the oocyte dysfunction caused by abnormal cell proliferation or apoptosis of CCs (9), consistent with results indicating that aberrant CC activity is associated with follicle maturation failure, anovulation, and infertility (9,10).

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Midkine (MK) is a 13 kDa cysteine-rich protein that is developmentally vital as a retinoic acid-responsive gene product during mid-gestation (11). MK is thus categorized as a heparin-binding protein, belonging to the family of heparin-binding growth associate molecules (HB-GAM) (12). MK is prominently expressed during embryogenesis but down-regulated to negligible levels in healthy adults (13). MK acts through several plasma membrane receptors, including syndecans, integrins, protein tyrosine phosphatase ζ (PTPζ), anaplastic lymphoma kinase (ALK), low-density lipoprotein (LDL)-receptor-related protein (LRP), and Notch2 receptors (14). It has important functions related to growth, proliferation, survival, migration, angiogenesis, reproduction, and repair (14). Previous reports showed that it is also a key player in autoimmune disorders (i.e., Multiple Sclerosis), ischemia, inflammation, and most notably various cancers (14). The apoptosis-suppressing effect of MK on several cells, such as cumulus cells, neuronal cells, and cancer cells, etc. (15-17).

Previous reports showed that HYAse treatment of human oocytes led to decreased oocyte survival, fertilization rates, and developmental rates following intracytoplasmic sperm injection (ICSI) application (18-20). In addition, it has also been shown in mouse models that longer exposure to HYAse decreases the quality of mouse oocytes, and shorter HYAse application may lead to a higher fertilization rate in fresh and cryopreserved oocytes (21). The role of MK in reproduction has been understudied (16,22-24). In these studies, it was shown that MK increased the proliferation of primordial germ cells (PGCs) through the inhibition of apoptosis, and MK promoted the in vitro maturation (IVM) of oocytes via its anti-apoptotic effects on the cumulus-granulosa cells (16,22). MK targets directly CCs rather than oocytes for its developmental competence-promoting effects (23,24).

Taken together, the study aimed to investigate the mechanisms underlying CC dysfunction through the identification of MKs' role during the related denudation process.

Material and Method

Patients profile: study proceeded as a prospective, randomized study. COCs were taken from women during their first cycle of IVF using ICSI in the IVF Center at the private hospital in Istanbul from September 2017 to September 2019. This study was conducted according to the principles of the Declaration of Helsinki, and all patients signed and provided written informed consent before enrollment in the study. The study was reviewed and approved by the ethics committee of Biruni University (Ethics approval reference number: 2017/5-1).

All patients donated their CCs after providing written informed consent. Cumulus cells were obtained from a total of 90 women aged 21-40 and diagnosed as male factor infertility via a mechanical application. Patients older than 40 years or younger than 21 years, and those with a history of smoking, endometriosis, endocrine disease, diabetes, obesity, alcohol consumption, vitamin D or A use, oral contraceptive use, surgery, radiation or chemotherapy, or inflammatory diseases were excluded from this study.

Cumulus cell culture: Human cumulus cells were dissected from the COC obtained from a total of 90 women undergoing ICSI using a sterile needle. The cells were dispersed by gentle pipetting-mechanical denudation-without using hyaluronidase enzyme. Isolated cumulus cells cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in ambient O_2 and 5% CO_2 in a humidified incubator.

Experiment design: For subsequent experiments, proliferated cells were seeded at 6-well plates containing 5 mL DMEM-F12 medium at a concentration of 500.000 cells (25). Most commercially available hyaluronidases have a concentration of 80 IU/L, which is only one-tenth of the critical threshold required for parthenogenetic activation (26). Hyaluronidase enzyme concentrations of 0.1 IU (the lowest concentration), 1 IU, and 10 IU (the highest concentration) were applied to CCs. They were cultured in these enzyme concentrations for 48 hours. At 24-hour intervals over 48 hours, total cell numbers, as well as viable, apoptotic, and dead cell rates were determined using the flow cytometric Annexin-V-Fluorescein isothiocyanate/propidium iodide (Annexin-V-FITC/PI) double staining (25). MK levels with Enzyme-Linked ImmunoSorbent Assay (ELISA) and the ultrastructure of CCs with transmission electron microscopy (TEM) were determined.

The analysis of viability and apoptosis: To determine the viable, apoptotic, and dead CCs along with the total cell number, Annexin V-FITC/PI double staining was evaluated using the FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, San Diego, CA) (25). Single-cell suspensions of CCs were washed twice with cold phosphate-buffered saline (PBS) and then resuspended in 100 µL × binding buffer (0.01 M HEPES, 0.14 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. CCs were stained for 15 min with 5µl Annexin V-FITC and propidium iodide (PI) at room temperature (RT) in the dark and analyzed by flow cytometry (BD Bioscience FACS Calibur, San Diego, CA) within 5-10 min (25). At least 10.000 cells were analyzed per patient. The percentages of viable, early apoptotic, late apoptotic, and dead cells were calculated using the CellQuest and WinMDI analysis programs (25).

The analysis of midkine protein levels: The determination of protein concentrations: CCs were lysed and protein concentrations of each lysate were determined by the Bradford Method using Pierce™ Detergent Compatible Bradford Assay Kit (Catalog no: 23246; Thermo Scientific, Germany) and following the kit's described microplate micro protocol with a

sensitivity range of 2-25µg/mL. As described in the instruction manual of the kit, 150 µl of each bovine serum albumin standard and samples were pipetted into the microplate wells. 150 µl of Pierce Detergent Compatible Bradford Assay Reagent was added to each well with a multi-channel pipettor. The plate was incubated for 10 minutes at room temperature (RT). The absorbance at 595 nm was measured using a plate reader. The protein concentration was determined in µg/mL using the standard curve.

The analysis of midkine protein levels: MK levels were detected using an industrial ELISA kit (Catalog no., CDYELISA; Cellmid Limited, Sydney, Australia), according to the manufacturer's instructions with some minor modifications (25). Briefly, 100 µl/well of samples (The supernatants of lysed CCs), two positive controls (one of them supplied by the ELISA kit, and the second one obtained from T98 glioblastoma cells, which have been previously reported to exhibit high MK levels), and the standards (The concentrations ranging from 0 to 1000 pg/mL were obtained by diluting MK master standard in a concentration of 107 pg/mL) were all incubated at RT for 2 h with continuous shaking on an orbital shaker. All standards, controls, and samples were used in triplicate. After every step except for the application of the stop reagent, three washing applications using the washing buffer supplied by the kit were performed. Detector antibody (100 µl/well) was applied and wells were incubated with that antibody at RT for 1 h. The samples were incubated with 100 µl/well streptavidin-peroxidase solution for 25 min and 100 µl/well Substrate Solution for 20 min in the dark at RT. After 15 min, 100 µl/well stop solutions were added to inactivate the enzyme and detect yellow color formation. At last, the results were determined at a wavelength of 450 nm using a Multiskan Go ELISA microplate reader (Thermo Scientific, Germany) within 5 min.

The ultrastructure analysis: Briefly, CCs were fixed for 1 day in 2.5% glutaraldehyde (Sigma Chemical Co, St. Louis, MO, USA)/Sodium Cacodylate Buffer at 4°C, embedded in 1% agar, then post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, 1.560 Industry Road, Hatfield, PA, USA) (27). The samples were dehydrated in increasing concentrations of ethanol (Sigma Chemical Co, St. Louis, MO, USA) and then immersed in propylene oxide (Sigma Chemical Co, St. Louis, MO, USA) for solvent substitution. CCs in agar were embedded in EMbed-812 (Electron Microscopy Sciences, Hatfield, PA, USA). Then, the blocs of CCs were sectioned for TEM evaluations. The cells were sectioned at a thickness of 0.5-1 µm for light microscopy and stained with toluidine blue (27). After light microscopy evaluation, ultrathin sections of 60-80 nm were cut with a diamond knife, mounted on nickel grids, and contrasted with saturated uranyl acetate and lead citrate (Electron Microscopy Sciences, Hatfield, PA, USA) before being examined and photographed using Jeol-Jem 1011 electron microscopes (Electron Microscopy Sciences, Hatfield, PA, USA) operating at 80 kV. For each experimental group, at least 5 CCs were selected randomly for morphometric analysis (27).

Statistical analysis

The data presented are the mean and standard deviation (SD) of 3 separate experiments done in duplicate. The determination of significance between groups was done by using SPSS software (Version 24) according to one-way analysis of variance (ANOVA) with post hoc Tukey tests for multiple comparisons. Graphs were drawn by using Microsoft Excel 2019. A p-value lower than 0.05 was considered statistically significant.

Results

Table I shows the demographic data. In addition to the exclusion criteria, the data confirmed that cumulus cells were taken from healthy subjects.

Table I: *The demographic data*

Variables	Group
Age	31.65 ± 5.22
BMI	23.60 ± 2.56
Endometrium thickness (mm)	11.00 ± 1.00
AFC	7.20 ± 3.00
Oocyte number	8.81 ± 3.73
GV	0.50 ± 0.25
MI	0.25 ± 0.25
MII	5.24 ± 2.43
Fertilized oocyte number	4.82 ± 2.76
Fertilisation rate (%)	54.04 ± 5.4
Embryo number	4.48 ± 2.41
Good quality (Grade I) embryo number (Day 3)	2.28 ± 0.82 (51%)
Good quality (Grade II) embryo number (Day 3)	0.95 ± 0.91 (21%)
Bad quality (Grade III) embryo number (Day 3)	0.89 ± 0.22 (19%)
Bad quality (Grade IV) embryo number (Day 3)	0.44 ± 1.11 (9%)
Good attachment (%)	43.5%
Pregnancy rate (Sac+) (%)	29.60 ± 4.53

AFC: Antral follicle count, BMI: Body mass index, GV: Germinal vesicle (Blocked oocyte maturation at the prophase stage of meiosis), MI: 1. Meiotic metaphase, MII: 2. Meiotic metaphase.

Cumulus cell proliferation: As clearly shown in Figure 1, all enzyme applications inhibited cell proliferation for all hours in comparison to the control group (The 24th h: $p_{0.1}>0.05$, $p_1>0.05$, $p_{10}>0.05$; The 48th h: $p_{0.1}<0.01$, $p_1<0.001$, $p_{10}<0.01$). Although there was no prominent difference between the test groups, the control group, and the same test group members at the 24th h, the difference became prominent at the 48th h. The highest inhibitory effect was determined at the high enzyme concentration at the end of the 48th h ($p_{10}<0.01$), and the lowest inhibitory effect was found at the low enzyme concentration at the end of the 48th h ($p_{0.1}<0.01$).

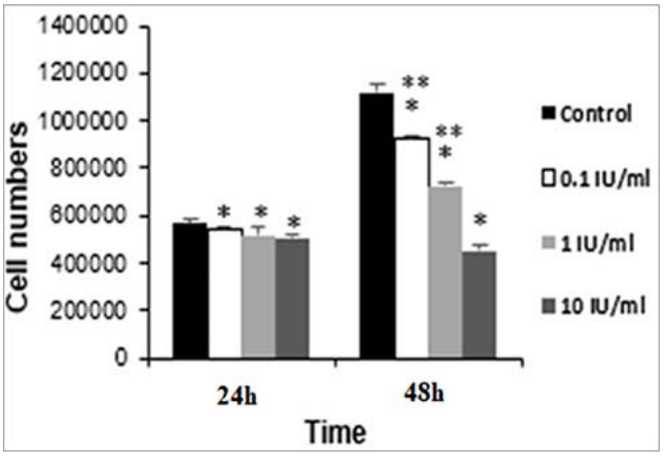

Figure 1: *The alterations at cell proliferation index. *p<0.05 in comparison to the control group, **p<0.05 in comparison to the high concentrated group*

Cell viability and apoptotic indices: The effects of different enzyme applications on cell viability are shown in Figure 2. All concentrations of enzyme decreased the cell viability for all hours in comparison to the control group (The 24th h: $p_{0.1}<0.05$, $p_1<0.05$, $p_{10}<0.05$; The 48th h: $p_{0.1}<0.05$, $p_1<0.05$, $p_{10}<0.05$). There was no prominent significance between test groups at the 24th h ($p>0.05$), however, the difference became prominent at the 48th h. The highest cell viability (53%) with the lowest apoptotic cell ratio (20%) and dead cell ratio (27%) was determined at the low enzyme concentration at the 48th h ($p_1<0.05$, $p_{10}<0.001$). The lowest cell viability (39%) with the highest apoptotic cell ratio (23%) and dead cell ratio (39%) was determined at the high enzyme concentration at the 48th ($p_{0.1}<0.001$, $p_1<0.05$).

MK protein levels: Figure 3 shows how different enzyme applications affected MK levels. Following results, all enzyme concentrations decreased MK levels for all hours in comparison to the control group (The 24th h: $p_{0.1}<0.05$, $p_1<0.05$, $p_{10}<0.01$; The 48th h: $p_{0.1}<0.01$, $p_1<0.001$, $p_{10}<0.00001$). For all hours, the highest decrease in MK levels was detected at the highest enzyme concentration (The 24th h: $p_{0.1}<0.001$, $p_1<0.05$; The 48th h: $p_{0.1}<0.0001$, $p_1<0.01$).

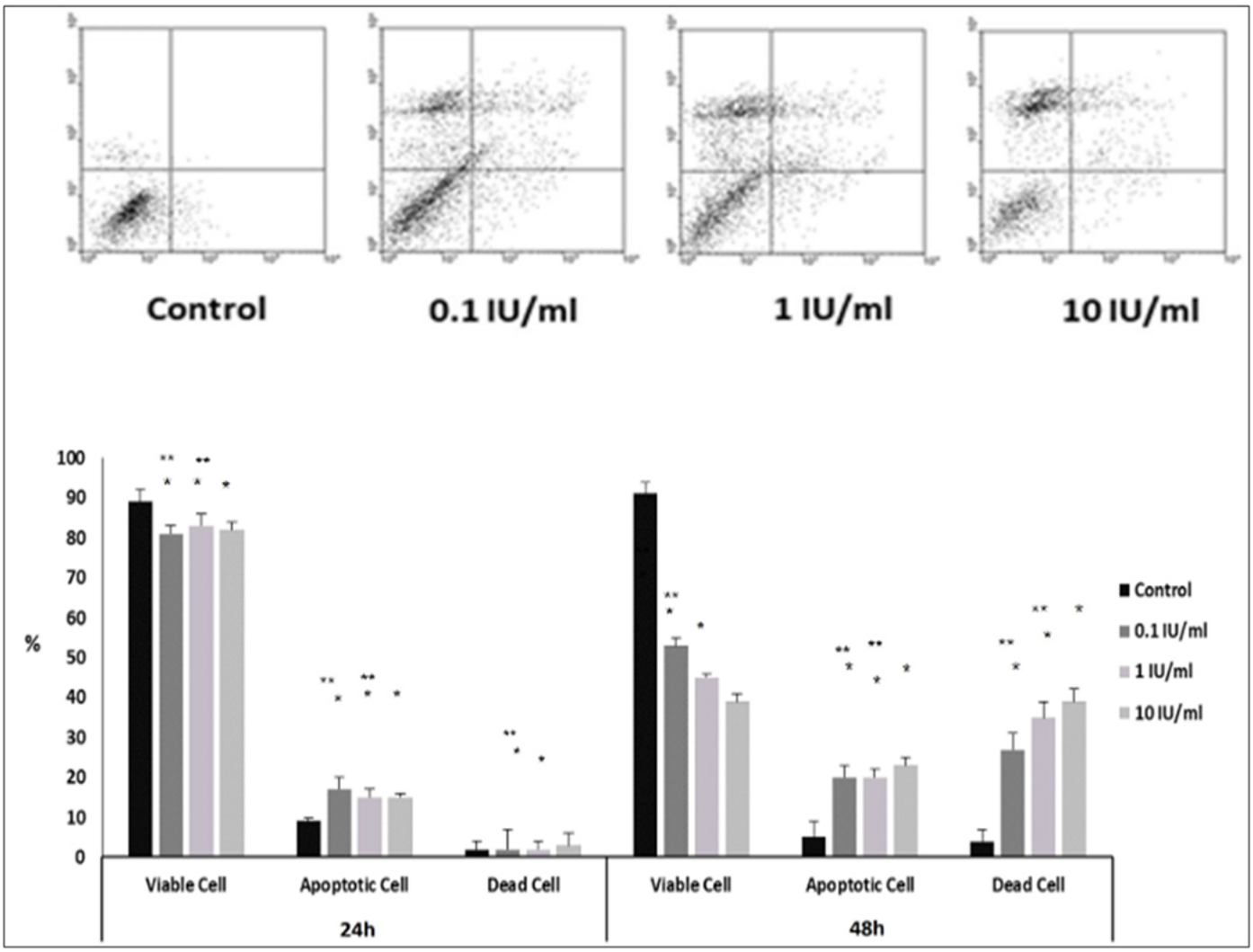

Figure 2: *The alterations in the number of viable, dead, and apoptotic cells. Figure 2 consists of a flow cytometric histogram at the 48th h and the graphs. *p<0.05 in comparison to the control group, **p<0.05 in comparison to the high concentration group.*

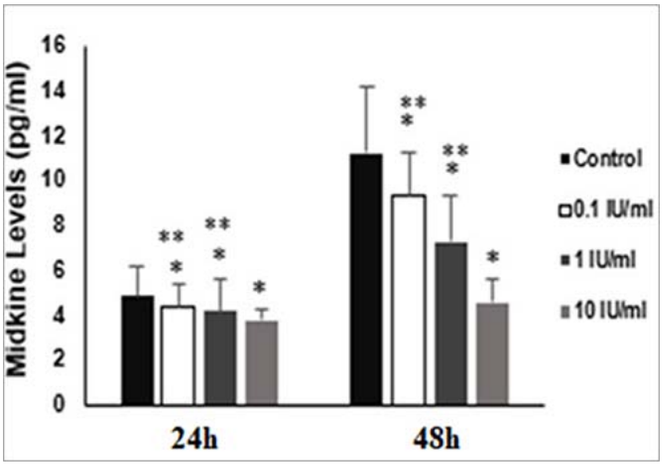

Figure 3: *The alterations at midkine levels. *:p<0.05 in comparison to the control group, **:p<0.05 in compasion to the high concentration group.*

The alterations in ultrastructure: TEM evaluations proceeded for the 48th hour. The control group showed healthy ultrastructure (Figure 4A). CCs were round or oval and surrounded by a continuous cytoplasmic membrane. Large oval nuclei were delimited by a continuous and electron-dense nuclear membrane. Spots of heterochromatin were clustered beneath the nuclear membrane. The organelles were abundant and uniformly scattered in the cytoplasm. Major organelles were those typical of steroidogenic cells, such as mitochondria and lipid droplets. In addition, cell remnants were rarely seen (Figure 4A). Some cells in the group with the lowest concentration of HYAse showed a healthy ultrastructure similar to the control group. In addition, some cells with an apoptotic cell appearance and apoptotic bodies were also observed, but they were found in low numbers (Figure 4B). Cells with a healthy ultrastructure were also observed; however, they were found in low numbers in the group with a mild concentration of HYAse. Cells with apoptotic appearance, cell remnants, and apoptotic bodies were detected in high numbers. Mild mitochondria damage was also found in a low number (Figure 4C). Cells with a healthy ultrastructure were detected very rarely in the group with the highest concentration of HYAse. Cells with apoptotic appearance, cell remnants, and apoptotic bodies were observed abundantly. In addition, cells with vacuolated cytoplasm were also detected in high numbers. Severe mitochondrial damage was also observed in very low numbers (Figure 4D).

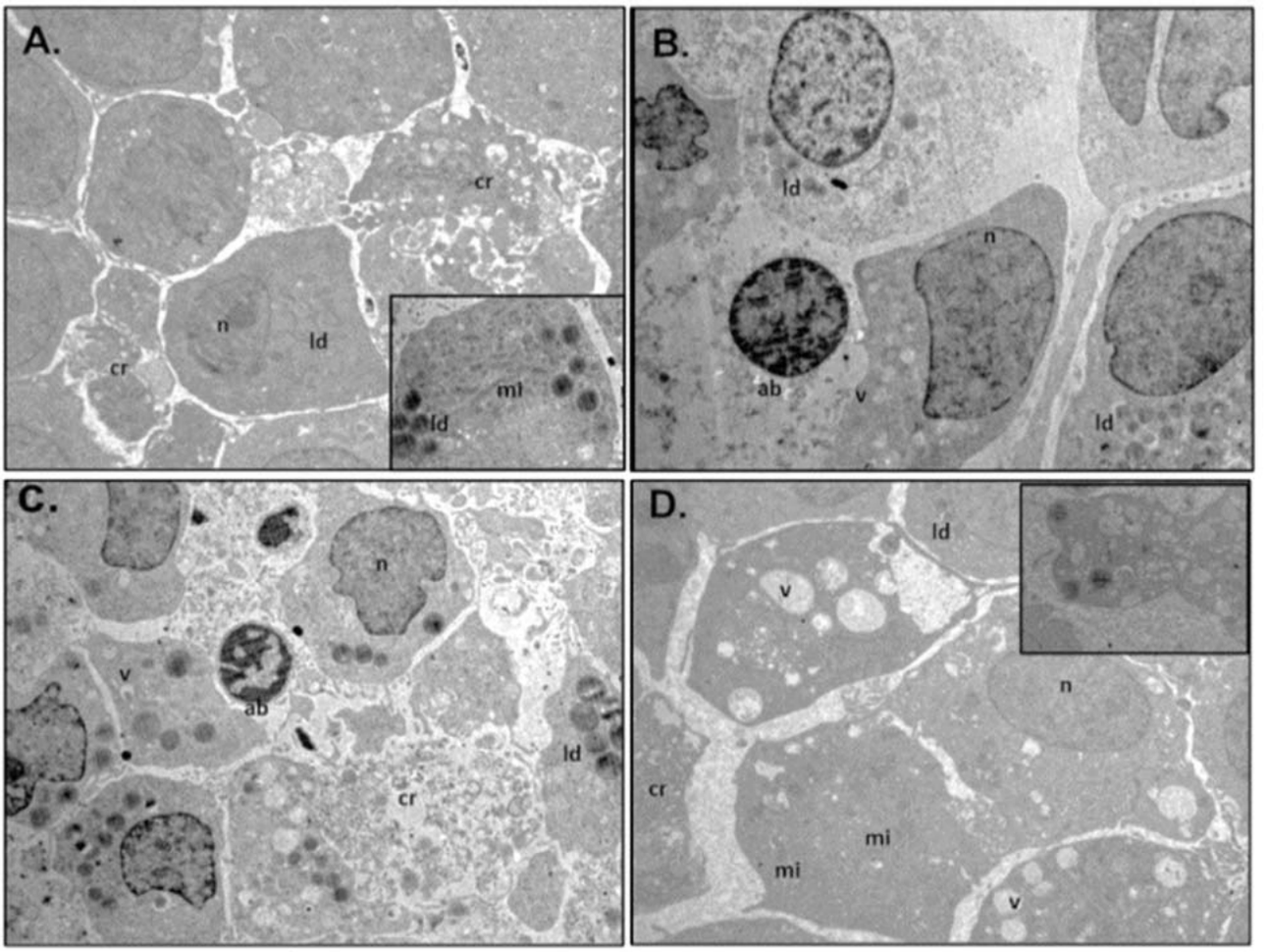

Figure 4: *The alterations at the ultrastructure of cumulus cells at the 48th hour. A: The control group (×5000), Small micrograph (×15k); B: 0.1 IU/mL (×5000), C: 1 IU/mL (×5000), D: 10 IU/mL (×5000), Small micrograph (x15k). mi: mitochondria, v: vacuole, ıd: lipid droplet, ab: apoptotic body, cr: cell remnant, n: nucleus*

Discussion

Ikeda et al. showed that cumulus cells of cumulus-enclosed oocytes (CEOs) spontaneously undergo apoptosis during IVM. They concluded that CCs are essential in IVM, and apoptosis may be related to the developmental competence of enclosed oocytes (28). Other publications support the findings of Ikeda et al. (29,30).

Ebner et al. conducted a prospective study with patients suffering from tubal factors, unexplained infertility, and more than one factor based on the comparison of denudation techniques (31). They showed that either cutting off supernumerous cumulus cells of COC or treating COC with hyaluronidase (The final concentration of 7 IU) has no difference in improving treatment outcome (31). They determined higher fertilization, compaction (Day 4), and blastocyst quality (Day 5) at the end of the HYAse application; however, they determined that implantation, pregnancy, and live birth rates are not different among any of the methods (31). Interestingly, they also reported that prolonged overnight HYAse (7 IU) incubation has no negative effect on further outcomes (31), and this finding is just the opposite of other studies (32) For example, Ishizuka et al. showed that prolonged HYAse exposure decreases the fertilization and development rates of fresh and cryopreserved oocytes in vivo (32) They treated oocytes with (0.1% HYAse (801 unit/ml) for 5 min or longer resulting in lower morula and blastocyst development. They also exerted that a longer exposure to HYAse reduces vitrified-warmed oocytes' survival, fertilization, and developmental rates (32) Finally, they concluded that shortened HYAse application times can lead to an increase in fertilization rate in both fresh and cryopreserved oocytes (32). In addition to the previously mentioned report by Ishizuka et al. (21), Montani et al. determined that HYAse leads to alterations in lipid composition such as phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine which are involved in the apoptosis process in CCs using HYAse according to the ICSI protocol commonly used in human reproduction clinics (33).

Human studies are not allowed by law. Therefore, we used human cumulus cells after the mechanical denudation process in our current study. We cultured the cells, and then we applied three different HYAse concentrations. As a result, we were unable to track the effects of these different enzyme applications on oocyte development, quality, fertilization, and pregnancy. This study can be conducted on animal models; however, the structure and metabolism of human CCs differ from those of animal CCs. In our study, we aimed to interpret our results for human beings.

Three different HYAse concentrations 0.1, 1, and 10 IU were applied to human CCs, and the inhibition of CCs' proliferation by all groups was seen at the 48th hour prominetly. Our results are the exact opposite of the findings of Ebner et al. as our findings showed that prolonged application of any concentration of HYAse, including the low concentration (i.e., 0.1 IU), can lead to CC damage (31). The results of CC viability and apoptosis also support our findings about the proliferation of CCs. A prominent decrease in viability and an increase in apoptosis were also observed at the 48th h., along with decreased proliferation. The 10 IU concentration had the highest inhibitory effect on the proliferation, with the highest apoptosis rate and lowest viability rate. Our findings confirmed the detrimental effects of prolonged HYAse applications, as reported by Ishizuka et al (32). In addition to apoptosis analysis via flow cytometry, although we did not investigate caspase 8, caspase 9, and caspase 3 levels via ELISA, TEM evaluation guides us to think that HYAse exerts its inhibitory effect through mitochondria-related apoptosis (i.e., intrinsic apoptosis). A higher number of damaged mitochondria was found at the highest concentration of HYAse compared to other groups.

Previous studies done by Ikeda et al. exerted that MK enhanced the viability of CCs in CEOs at IVM via the inhibition of apoptosis (16,23,24). In addition, the targeted inhibition of MK by several agents leads to apoptosis, thus MK can be accepted as a survival/resistance factor for several illness cases, such as cancer (34-38). In our study, HYAse in all concentrations inhibited MK levels for all hours. The inhibition of MK at the 48th hour was higher than the inhibition of MK at the 24th hour. The highest inhibitory effect was determined at the highest concentration of HYAse. The inhibitory effect of HYAse on MK was decreased from high concentration to low concentration, and this finding is also parallel to the decrease in cell proliferation and cell viability with the increase in apoptosis. Our results also prove that the inhibitory effect of HYAse on MK can be both time and concentration-dependent.

The limitations of the study include the inability to evaluate the effect of HYAse on CCs in women under 21 and over 40 years of age. Therefore, the overall resistance and fragility of CCs to these HYAse concentrations could not be evaluated. In addition, the HYAse effects should be evaluated in cases of female infertility, such as PCOS. The strength of this study is that it is the first report to examine the effect of HYAse on MK activity. 2-3 lines of CCs are commonly left during oocyte denudation to protect the oocyte from stress-induced reactions. The low number of CCs means low MK levels resulting in a loss in oocyte competence. This study explains one of the possible mechanisms of oocyte incompetency via midkine levels in cumulus cells.

In conclusion, the inhibitory effect of HYAse is time-dependent and concentration-dependent. Prolonged HYAse incubation increases its capacity to cause harm. MK can be involved in the HYAse mechanism of action. MK levels can indicate HYAse-related CC damage; thus, this can provide projections about oocyte quality.

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Ethics approval and consent to participate: All participants signed informed written consent before being enrolled in the study. The study was reviewed and approved by the ethics committee of Biruni University (Ethics approval reference number: 2017/5-1 date 26.04.2017). All procedures were performed according to the Declaration of Helsinki.

Availability of data/materials: The data supporting this study is available through the corresponding author upon reasonable request.

Authors' Contributions: ME participated in conception and design, experiments, conducted data collection and analysis, interpretation, commentary, and writing; EA participated in experiments, data collection, and analysis; TI participated in experiments, conducted data collection and analysis, and supervised. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that they have no competing interests.

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