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Research Article

Correlation between Oocyte Morphology and Developmental Stages for Human Oocytes that Failed to Cleave after Intracytoplasmic Sperm Injection

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Abstract

Background: In the context of intracytoplasmic sperm injection (ICSI), oocyte maturity is estimated by the oocyte's appearance only, which may not reflect its actual maturity. ***Objectives***: This study aims to compare the morphology of oocytes that failed to cleave after ICSI with the developmental status at which they were arrested. ***Methods***: Human oocytes that failed to cleave after ICSI were collected from in vitro fertilization cycles. Oocytes were examined and photographed under a light microscope for morphological study, then immunostained to visualize the chromatin material (of the oocyte and the injected sperm) and the meiotic spindle. The morphologic features were compared with the oocyte developmental stage to identify any possible correlation. ***Results***: 193 failed oocytes were collected, of which 182 were successfully immunostained and analyzed. Oocyte measurements did not differ significantly in relation to the developmental stage. Fine oocyte cytoplasmic granulation was connected with better oocyte maturity; dispersed granulation was connected with lower maturity levels, while central granulation was connected with the lowest oocyte maturity. A single polar body was significantly associated with early oocyte developmental stages, while two polar bodies were significantly associated with later developmental stages. Meiotic spindles were confined to metaphase-II oocytes. Oocyte morphology and measurements had no relation to the degree of sperm chromatin condensation. ***Conclusions***: For the oocytes that fail ICSI, morphology correlates with the developmental stages. Fine cytoplasmic granulation is connected with better oocyte maturity, while dispersed and central granulation patterns are connected with lower maturity.

Keywords: Developmental stage; Fertilization failure; Meiotic spindle; Oocyte.

العلاقة بين شكل البويضة ومراحل النمو لدى البويضات البشرية التي فشلت في الانقسام بعد حقن الحيوانات المنوية داخل السيتوبلازم

الخلاصة

الخلفية: في سياق حقن الحيوانات المنوية داخل السيتوبلازم (ICSI)، يقدر نضج البويضة فقط من خلال مظهر البويضة، والذي قد لا يعكس نضجها الفعلي. **الأهداف**: مقارنة شكل الخلايا البويضية التي فشلت في الانفصال بعد ICSI مع الحالة التطورية التي وصلت إليها. **الطرائق**: أجريت دراسة مقطعية رصدية على بويضات بشرية فشلت في الانقسام بعد ICSI وجمعت من دورات الإخصاب الصناعي. تم فحص وتصوير البويضات تحت المجهر الضوئي للدراسة الشكلية، ثم صبغها المناعي لرؤية مادة الكروماتين (من البويضة والحيوانات المنوية المحقونة) والمغزل الاختزالي. تمت مقارنة السمات الشكلية مع مرحلة تطور البويضات للعثور على أي ارتباط محتمل. **النتائج**: تم جمع 193 بويضة فاشلة، وتم تلوينها وتحليلها مناعيا 182؛ تلفت أحد عشر بويضة خلال الإجراء. لم تختلف قياسات البويضات بشكل كبير بالنسبة لمرحلة التطور. تم ربط الحبيبات السيتوبلازمية الدقيقة للبويضات بنضج أفضل للبويضة؛ كانت الحبيبات المتفرقة مرتبطة بمستويات نضج أقل، بينما كانت الحبيبات المركزية مرتبطة بأدنى نضج للبويضات (p=0.006). ارتبط الجسم القطبي الواحد بشكل كبير بالمراحل المبكرة لتطور البويضات، بينما كان وجود جسمين قطبيين مرتبطا بشكل كبير بالمراحل التطورية اللاحقة (p=0.000). كانت مغازل الانقسام مقتصرة على بويضات الطور الاستوائي الثاني. لم يكن لشكل وقياسات البويضات علاقة بدرجة تكثف كروماتين الحيوانات المنوية. **الاستنتاجات**: بالنسبة للبويضات التي تفشل في ICSI، قد يرتبط الشكل بمراحل النمو. أظهرت الحبيبات السيتوبلازمية الدقيقة ارتباطا بنضج أفضل للبويضات، بينما أظهرت أنماط الحبيبات المتفرقة والمركزية ارتباطا بنضج أقل.

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INTRODUCTION

Since the birth of the first human following the intracytoplasmic sperm injection procedure, ICSI has become the gold standard treatment offered for severe cases of infertility. However, fertilization is never guaranteed despite the actual introduction of sperm into the oocyte. Fertilization failure is attributed to reasons related to the oocyte, the sperm, and both. Often, fertilization failure occurs even when the oocyte and the injected spermatozoon are apparently normal. This indicates that the mere morphology of gametes does not necessarily ensure functional competence. In fact, many studies performed on apparently metaphase-II (MII) human oocytes showed that these oocytes have not reached MII stage by the time of ICSI [1]. In IVF laboratories, an oocyte's morphological features can be identified under the light microscope. These include oocyte measurements, the presence and number of polar bodies (PBs), germinal vesicles, perivitelline space (PVS), and oocyte cytoplasmic features like granulation, vacuoles, smooth endoplasmic reticulum (SER) aggregates, and refractile bodies (RBs). However, the exact developmental stage of the human oocyte cannot be revealed by those morphological features and can only be determined by visualizing the chromosomes and meiotic spindle using methods like the immunofluorescence technique [1,2]. A large number of studies have investigated the relation between the presence of a meiotic spindle and IVF outcomes like fertilization rate, implantation rate, pregnancy, and live birth rates [2,3]. However, because visualizing the MS and chromosomes of the oocyte

requires the use of the immunofluorescence technique, which ends the vitality of the oocyte, only a limited number of studies have compared oocyte morphology to the developmental status as revealed by visualizing the MS and chromosomes. In the current study, we aimed to shed light on this aspect, which differs from previous studies by examining the oocyte's exact development status through the visualization of the MS and chromatin, rather than relying solely on the presence and number of polar bodies. In this study, we hypothesized that the oocyte's morphologic features, which are visible under a light microscope, may be influenced by its actual developmental stage, as revealed by visualizing the chromatin and meiotic spindle.

METHODS

Study design and setting

A laboratory-based cross-sectional observational study evaluating the association between the morphologic/morphometric oocyte features and the oocyte's developmental stage of maturity in the context of intracytoplasmic sperm injection.

Sample selection

The study involved oocytes from females having IVF treatment for infertility (primary or secondary) in the form of intracytoplasmic sperm injection (ICSI) at Kamal Al-Sameraie Hospital for Infertility Management, Baghdad, from July to October 2025. The study sample (oocytes) was collected during the second day following the insemination of the oocytes, as by this day any oocyte that doesn't show mitotic division is considered failed. 193 failed oocytes were collected initially from 97 women (2 oocytes per patient), from which 182 oocytes were successfully immunostained and evaluated. The remaining oocytes were destroyed during the immunofluorescent study procedure.

Inclusion criteria

Oocytes that failed to show the first cleavage by the second day after ICSI from females aged 37 years or younger were included in this study.

Exclusion criteria

We excluded all oocytes that did cleave by day 2 post-ICSI and excluded all oocytes from females ages 38 or older.

Intervention and outcome measurements

All females from which the oocytes were collected had controlled ovarian hyperstimulation using the antagonist protocol. The selected failed oocytes were examined and photographed on day 2 under an inverted light microscope with 400x magnification. Oocyte morphometric study included the measurements of oocyte cytoplasmic diameter, zona pellucida thickness, first polar body (PBI) size, and perivitelline space (PVS) size. The morphologic study included examining the cytoplasmic granulation pattern, number and shape of polar body(s), perivitelline space granulation, and other intracytoplasmic abnormalities like vacuoles and smooth endoplasmic reticulum aggregates (SER). Assessment of morphologic features was blinded and was done by a well-trained observer.

Immunofluorescent staining

The failed oocytes underwent immunofluorescent staining to examine the chromosomes and meiotic spindles. This was done by using a beta-tubulin monoclonal antibody, which visualizes beta-tubulin protein subunits in the microtubules making up the meiotic spindles. As for the chromatin material, it was visualized by using a special fluorescent stain (4',6-diamidino-2-phenylindole (DAPI)), which stains the DNA molecules in the cell. Oocyte fixation was done at 37°C by incubating them in 2% paraformaldehyde in phosphate-buffered solution (PBS) for a duration of 10 minutes, after which the oocytes were transferred to another phosphate-buffered solution with Tween-20 (PBST) and stored at 4°C in the refrigerator, waiting for the immunostaining step [4]. The immunofluorescence staining process was performed according to the following steps: the oocytes were taken out of the refrigerator and allowed to equilibrate with the room temperature, then they were put in 0.25% Triton X-100 in PBS for 15 minutes to achieve permeabilization (creation of holes in the cell membrane to allow for the entry of the other agents into the cell). Then the oocytes were transferred into a blocking solution that contains normal goat blocking buffer and left in the incubator for 30 minutes. After that step, the oocytes were exposed to the primary antibody by incubating them in anti-beta tubulin monoclonal antibody (Elabscience®, 1:100 dilution) for a total of one hour. Then the oocytes were washed three times in PBST, five minutes for each wash. Following this, the oocytes were incubated in goat anti-mouse IgG (FITC-conjugated) (Elabscience®, 1:50 dilution) as the secondary antibody for one hour in order to label the regions of attachment of the primary antibody. This was followed also by washing three times in PBST, and then the oocytes were counterstained by DAPI (0.1 mg/mL) for 20 minutes to visualize the chromatin material and washed in PBST. As a final step, each oocyte was individually mounted on a positively charged glass slide. The slides were prepared to preserve the three-dimensional structure of the oocyte. Anti-fade mounting medium (anti-fluorescence quenching agent, Elabscience®) was used to mount the oocytes, which were then covered with a cover slip and stored for immunofluorescence microscopic examination.

Ethical considerations

This study protocol was approved by the Research Ethics Committee of the College of Medicine, Mustansiriyah University. On the oocyte collection day, consent was taken from the females to use their oocytes that fail to cleave in this study.

Data analysis

After morphologic and immunofluorescence examination of the oocytes, the results were analyzed using SPSS software to detect possible correlations and associations and to confirm or deny statistical significance. Using SPSS (v. 26), data were examined for normal distribution using Shapiro-Wilk lot testing. Categorical data was expressed as frequency and compared to each other using the chi² test. Numerical data were expressed as mean ± standard deviation and were first examined using one-way ANOVA and then compared to each other using Tukey's HSD test at a 95% confidence interval ($p< 0.05$).

RESULTS

Immunofluorescence study revealed seven different developmental stages shown by the examined oocytes, with metaphase-II and anaphase-II occurring at the greatest frequencies [60 oocytes (33%) and 51 oocytes (28%), respectively] (Table 1).

Table 1: Oocytes developmental stages as indicated by nuclear maturity

Oocyte developmental stage	Anaphase I	Telophase I	Prometaphase II	Metaphase II	Anaphase II	1PN	2PN
Number	9	13	22	60	51	16	11
Percentage	5%	7.1%	12.1%	33%	28%	8.8%	6%

1PN: one pronucleus, 2PN: two pronuclei.

Other developmental stages included anaphase-I, telophase-I, prometaphase-II, one pronucleus (1PN), and two pronuclei (2PN). While light microscopy showed many morphologic features of the oocytes, like the zona pellucida, perivitelline space, polar body, and cytoplasmic features, immunofluorescent images of the same oocytes revealed—most importantly—the chromatin material (of the oocyte, polar body, and the injected spermatozoon) and the meiotic spindle (when present). These structures, unlike simple morphology, can indicate the exact developmental stage reached by the oocyte (Figure 1).

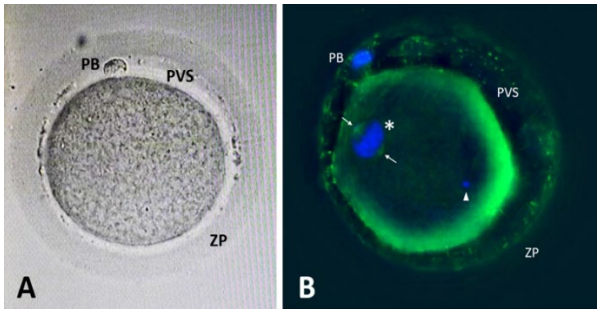

Figure 1: A: metaphase-II oocyte imaged under light microscope, showing the first polar body (PB), perivitteline space (PVS), and zona pellucida (ZP). The polar body is of average size and regular shape, and the cytoplasm shows dispersed granulation pattern (400x). B: metaphase-II oocyte images by immunofluorescence technique, showing the same structures as in A, plus the sperm chromatin (arrowhead), oocyte chromosomes (asterisk), and meiotic spindle (arrows). Chromatin material is shown in blue while cytoskeleton elements are shown in green (400x).

Oocyte measurements (oocyte diameter, ZP thickness, PBI size, and PVS size) did not differ significantly in relation to the developmental stage. Neither oocyte measurements nor morphological features had a significant difference in relation to the degree of sperm chromatin condensation. Some of the morphological features had a statistically significant association with some developmental stages and other immunofluorescence features of the studied oocytes. These can be summarized by the following: Different patterns of oocyte cytoplasmic granulation had a significant association with specific developmental stages of the oocyte (Table 2). Oocytes with a fine granulation pattern showed higher rates of reaching anaphase-II and 2PN stages compared to other granulation patterns. Oocytes with dispersed granulation patterns were associated with higher rates of metaphase-II and prometaphase-II stages. Oocytes with central granulation patterns were associated with higher rates of anaphase-I and telophase-I developmental stages, and oocytes with uneven granulation patterns were only associated with small rates of metaphase-II and anaphase-II. In other words, a fine granulation pattern was connected with higher levels of oocyte maturity, a dispersed granulation pattern was connected with lower maturity levels, and a central granulation pattern was connected with the lowest oocyte maturity levels. A regular, unfragmented first polar body (PBI) was seen in all of the oocytes that were in telophase-I and prometaphase-II, while fragmented PBIs were seen in some oocytes from the more advanced developmental stages (metaphase-II, anaphase-II, 1PN, and 2PN) (Table 2).

Table 2: Association of oocyte morphologic parameters with the developmental stage

Parameter	Status	Nuclear maturity stage (%)							*p*-value
		Anaphase I	Telophase I	Prometaphase II	Metaphase II	Anaphase II	1PN	2PN	
PB shape	Fragmented	25	0.0	0.0	37.5	39.4	16.7	33.3	0.038
	Regular	75	100	100	62.5	60.6	83.3	66.7	
2nd PB	Present	0.0	0.0	0.0	0.0	0.0	83.3	77.8	<0.001
	Absent	100	100	100	100	100	16.7	22.2	
PVS fragmentations	Present	0.0	40	64.3	37.5	60.6	66.7	11.1	0.026
	Absent	100	60	35.7	62.5	39.4	33.3	88.9	
Oocyte granulation pattern	Fine (normal)	0.0	30	35.7	45.8	63.6	16.7	55.6	0.006
	Central	75	50	21.4	16.7	0.0	0.0	11.1	
	Dispersed	25	20	42.9	33.3	33.3	66.7	33.3	
	Uneven	0.0	0.0	0.0	4.2	3.0	16.7	0.0	

Values are presented as percentage. 1PN: one pronucleus, 2PN: two pronuclei, PB: polar body, PVS: perivitelline space.

The presence of a single polar body (PBI) was significantly associated with the developmental stages of anaphase-I, telophase-I, prometaphase-II, metaphase-II, and anaphase-II. The presence of 2 polar bodies (PBI and PBII) was significantly associated with the developmental stages of one pronucleus (1PN) and two pronuclei (2PN) (Table 1). The presence of the meiotic spindle was confined to the metaphase-II developmental stage. Meiotic spindles were absent in all MII oocytes that showed condensed sperm chromatin and in 56% of MII oocytes with partially decondensed sperm chromatin. The presence of meiotic spindles was associated with significantly larger 1st PB sizes (Figure 2). Also, the presence of meiotic spindles was significantly greater in samples with a fine oocyte cytoplasmic granulation pattern (Figure 3).

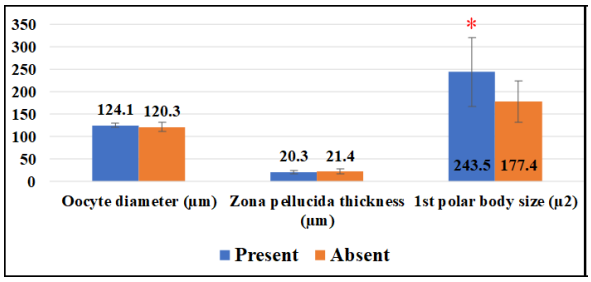

Figure 2: Association of meiotic spindle presence with the measurements of the oocyte. Sample size: 182, ANOVA and Tukey's *post hoc* tests. * statistically significant (p= 0.037) for first polar body size.

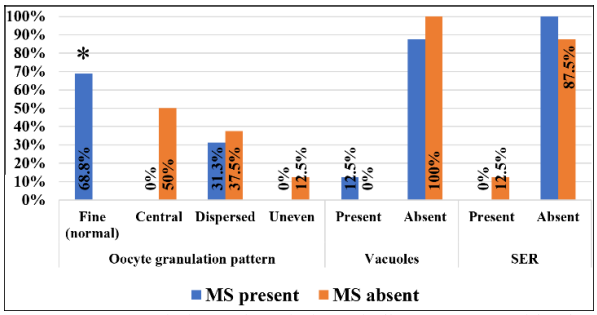

Figure 3: Association of meiotic spindle presence with the morphological features of the oocyte. Sample size: 182, Chi square test. *statistical significance at p= 0.001 for fine granulation.

DISCUSSION

It is well documented that the developmental stage of the oocyte is largely determined by changes related to the chromosome/chromatin of the gametes. Thus, the absence of oocyte morphometric changes in relation to the developmental stage in our results goes well with the published literature in this regard [5]. The morphometric oocyte parameters included oocyte diameter, ZP thickness, PBI size, and PVS size, all of which are not connected to the changes taking place on the chromatin level in the previous studies [6]. As for the cytoplasmic granulation, oocyte cytoplasmic texture is a highly important parameter of oocyte quality and developmental potential, and fine granulation has been connected to better IVF outcomes in many studies [7-12]. A normal MII human oocyte should have a light cytoplasm with fine- to moderate granulation [11,13]. In our study, oocytes with fine cytoplasmic granulation were found to have reached more advanced developmental stages (anaphase-II and 2PN) compared to the other oocytes, followed by oocytes with uneven granulation, which were associated significantly with metaphase-II and anaphase-II. In other words, oocytes having fine and uneven cytoplasmic granulation achieved more advanced developmental stages. These results strongly agree with many old and recent studies that report better IVF outcomes with such oocytes [14-16], and specifically with Hu *et al.* (2021) [16], who showed that oocytes with fine granulation were associated with the highest 2PN rate, a finding similar to our results. On the other hand, in our study, oocytes with central and dispersed cytoplasmic granulation were significantly associated with lower developmental stages (anaphase-I and telophase-I for central granulation, prometaphase-II and 1PN for dispersed granulation). The literature reveals a contradiction between the studies regarding the negative effects of central and dispersed cytoplasmic granulation on the IVF outcomes. Our results agree with the majority of studies describing poor outcomes associated with central and dispersed granulation in the ooplasm [14-17] and disagree with a few other studies that reported no effect of such cytoplasmic granulation patterns on the oocyte developmental outcomes [12,18]. The central cytoplasmic granulation pattern is connected to different mechanisms that need more research to confirm or deny. The most accepted theory in this regard is cytoplasmic maturation asynchrony, where the nucleus is pushed to faster maturation by the effect of the drugs used in controlled ovarian hyperstimulation during IVF cycles, leaving less time for the cytoplasm to redistribute its organelles properly. It is largely accepted that the finding of first polar body fragmentation is rather associated with the time elapsed from oocyte denudation and ICSI [19,20]. Our findings in this regard confirm the results of these studies, despite contradicting other studies that deny a possible connection between PBI morphology and oocyte developmental potential [14,19,21,22,23]. The presence of a visible first polar body (PBI) was one of the parameters that necessitated the insemination of the oocyte. Oocytes with one PB in our study were found to be in anaphase-I, telophase-I, prometaphase-II, metaphase-II, and anaphase-II. This finding is rational as it goes with the normal developmental progress of the human oocyte, which extrudes the second polar body only after fertilization. As for anaphase-II oocytes, PBII was not yet visible as the cytoplasm restriction was just beginning. PBII was visible in all oocytes of 1PN and 2PN stages, as these are post-fertilization stages. The meiotic spindle of the second meiotic division was confined to MII oocytes. However, it was absent in all of the MII oocytes that had condensed sperm chromatin and in half of the MII oocytes that had only partial decondensation of sperm chromatin. Many studies relate sperm chromatin decondensation to oocyte factors, among which cytoplasmic and nuclear maturity are mainly considered [24,25]. Our findings in this regard agree with these studies, as MS presence inversely correlated

with the sperm chromatin decondensation level of the oocytes. There was a significant association between MS presence and fine cytoplasmic granulation of oocytes. Since the literature considers cytoplasmic granularity (other than fine granulation) an indicator of cytoplasmic immaturity, our findings further support the studies that hypothesize a synchronization between nuclear and cytoplasmic maturation of the oocyte [6]. The presence of a regular, birefringent meiotic spindle (MS) in the retrieved oocytes is agreed to be of vital importance to the oocyte developmental capacity [26]. As for the measurement of polar body size, there are no references for a normal range of the PBI size; the literature only mentions a "large" polar body as an abnormality that may indicate meiosis-I errors [6]. In our study, MII oocytes that had no MS had smaller PBI, which may indicate aberrations in the meiosis-I cytokinesis. Our study included only oocytes that failed to cleave following ICSI; however, this limitation should not be interpreted as bias in sample selection because the study aims to investigate any possible correlation between the morphology and the developmental stage at which the oocyte was arrested following insemination. It is well known that oocytes that successfully cleave pass through all developmental stages without arrest, making them irrelevant to the aims of this study.

Study Limitations

The main limitations of this study include performing it in a single center and the sample size, which can be increased in similar future studies to maximize the result reliability. In addition, observer-dependent assessment can be considered one of the limitations. Also, this study was limited by the relatively small percentage of the failed oocytes compared to the larger number of successful oocytes that cleave and develop to embryos within each IVF cycle.

Conclusions

For the oocytes that fail ICSI, some morphological features correlate with the developmental stages of those oocytes. Better oocyte maturity may be linked to fine oocyte cytoplasmic granulation, while lower maturity appears to be associated with dispersed and central granulation patterns. Oocytes in early developmental stages have a single polar body, while oocytes in later developmental stages have two polar bodies. Only metaphase-II oocytes have meiotic spindles. Neither oocyte measurements nor morphological features had a significant difference in relation to the degree of sperm chromatin condensation.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request.

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