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ASSISTED REPRODUCTION TECHNOLOGIES

The effect of immature oocytes quantity on the rates of oocytes maturity and morphology, fertilization, and embryo development in ICSI cycles

Iman Halvaei · Mohammad Ali Khalili · Mohammad Hossein Razi · Stefania A. Nottola

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Abstract
Purpose The goal was to evaluate the role of the number of retrieved immature oocytes on mature oocyte counts and morphology, and also the rates of fertilization and embryo development in ICSI cycles.
Methods 101 ICSI cycles were included in this prospective evaluation. Patients were divided into 2 groups of A (≤ 2 immature oocytes) and B (> 2 immature oocytes). In sub-analysis, the impacts of the number of GV and MI oocytes were assessed on the rates of fertilization and embryo development. Also, correlations between the numbers of immature and mature oocytes, as well as maternal age between two groups were analyzed. Assessments of oocyte morphology, fertilization, embryo quality and development were done accordingly.
Results There was no correlation between the immature oocytes quantity with the number of mature ones. There were insignificant differences for embryo development between two groups, but fertilization rate was higher in group A (P=0.03). In sub-analysis, insignificant differences were observed between two groups of≤and >2 GV and MI oocytes for rates of fertilization and embryo development. Also, the rates of clinical pregnancy and delivery were insignificant between groups. The rate of morphologically abnormal oocytes had no significant difference between two groups, except for wide perivitelline space (PVS) which was higher in group A (P=0.03). There was no significant difference for maternal age between two groups.
Conclusions In cases with few retrieved immature oocytes, rates of fertilization and incidence of wide PVS may increase, although immature oocytes may not have any negative impacts on early embryo development, or the rates on number of mature oocytes.

Keywords Immature oocytes · Oocyte morphology · Fertilization rate · Embryo quality · ICSI

Abbreviations

ICSI	Intracytoplasmic sperm injection
ART	Assisted reproductive technology
COH	Controlled ovarian hyperstimulation
GV	Germinal vesicle
MI	Metaphase I
IVM	In-vitro maturation
GnRH	Gonadotropin releasing hormone
rFSH	Recombinant follicle stimulating hormone
hCG	Human chorionic gonadotropin
mm	Millimeter
IU	International unit
im	Intramascular
h	Hour
OBGYN	Obstetric and gynecology
WHO	World health organization

Capsule In cases with less than two retrieved immature oocytes fertilization rate may increase in cohort oocytes but has no effect on early embryo development. Also there is no correlation between the number of immature and mature oocytes in each cycle.

I. Halvaei · M. Ali Khalili (✉) · M. H. Razi
Yazd Institute for Reproductive Sciences,
Shahid Sadoughi University of Medical Sciences,
Yazd, Iran
e-mail: khalili59@hotmail.com

I. Halvaei
e-mail: ihalvaei@gmail.com

S. A. Nottola
Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, La Sapienza University of Rome,
Rome, Italy

2PN	Two pronuclei
SE	Standard error
OR	Odds ratio
CI	Confidence interval
PVS	Perivitteline space
MII	Metaphase II
AMH	Anti-Mullerian hormone
PB	Polar body
ZP	Zona pellucida
SERc	Smooth endoplasmic reticulum cluster
NS	Not significant

Introduction

Introduction of intracytoplasmic sperm injection (ICSI) was a great achievement to give the opportunity of having babies primarily for couples with male factor infertility [25]. In ART, controlled ovarian hyperstimulation (COH) is necessary for inducing the recruitment of multiple follicular development for harvesting the numerous healthy mature oocytes. In recent years, several COH protocols have been introduced to optimize the ICSI outcomes. Despite optimizing the COH protocols, approximately 20 % of the oocytes remain immature at the GV or MI stages [26].

One of the factors that may affect the ICSI outcomes is related to the number of retrieved oocytes [20,37]. In addition, the maturity of retrieved oocytes is important for the success of in vitro fertilization, because mature oocytes are used for ART; while, the remaining immature ones are generally discarded. Although, it is possible to mature these oocytes using in-vitro maturation (IVM) technology, but both pregnancy and implantation rates have been reported very rare [19,24]. In recent years, some investigators tried to find some predictive values for the number of retrieved immature oocytes in COH program administered for ART outcomes [14]. There are some predictors for the number of retrieved immature oocytes, such as the number of 2 to 6-mm antral follicles, ovarian volume and peak ovarian stromal blood flow velocity measured by doppler ultrasound during the follicular phase. Also, there have been direct relations between the number of immature oocytes and pregnancy rates following IVM of human oocytes [4,13,31]. However, there are no strict data about the probable impact of these immature oocytes on maturity and outcome of their mature cohort oocytes in ART program. It seems that the effect(s) of immature oocyte quantity on ICSI outcomes are scarce. Therefore, this study was designed to evaluate the role of the number of retrieved immature oocytes on the mature oocyte count, morphology, rates of fertilization and embryo development in ICSI cases.

Materials and methods

Patient selection

A total of 101 ICSI cycles with male factor infertility were included in this prospective study. The female age was between 19–44 years old (mean 31.6±0.2). Egg donation, natural cycles and IVM cases were excluded from the study. This study was approved by our institution ethics committee (number: 3706). For COH, the long protocol was used with GnRH agonist down-regulation, followed by rFSH (Gonal-F; Serono, Switzerland). The ovarian response was controlled by transvaginal ultrasound and serum estradiol concentration on the day of hCG injection. When diameter of at least two follicles was larger than 18 mm, 10,000 IU of hCG (i.m.; Profasi, Serono) was administered. Oocyte pick-up was performed 34–36 h after hCG injection under transvaginal ultrasound-guidance. All follicles larger than 14 mm were subjected to pick-up, while follicles smaller than 14 mm were not punctured. There was uniformity of the ultrasound measurements between our physicians.

Categorization of the immature oocytes

According to the number of immature oocytes, cycles were divided into two groups of A (≤ 2 immature oocytes) and B (>2 immature oocytes). Then, rates of fertilization and development of embryos were compared between two groups. In the sub-analysis, the impacts of the number of GV and MI oocytes were assessed on the rates of fertilization and embryo development. Also, the effect of maternal age on the quantity of immature oocyte was assessed.

ICSI procedure

Analysis of semen was performed according to WHO manual [33]. For sperm count and motility, Makler chamber and phase contrast microscopy were used. Percentages of progressive and non-progressive spermatozoa were reported.

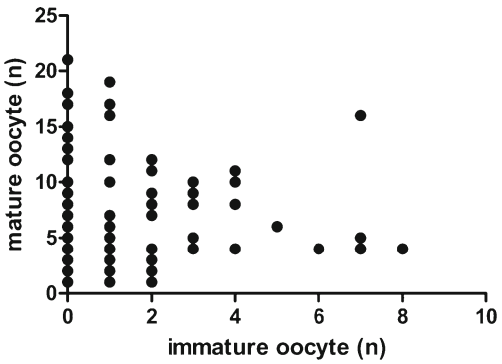

Fig. 1 Correlation between the number of immature and mature oocytes in each cycle

Table 1 Rates of fertilization, formation of good quality embryo, early cleaved embryo, pregnancy and delivery in two groups of patients

	Group A (≤2 immature oocytes)	Group B (>2 immature oocytes)	Odds ratio (95 % CI)	P-value
Number of cases	83	18		
Number of MII oocytes	509	121		
Fertilization rate (%)	320/509 (62.8)	63/121 (52)	1.56 (1.05–2.33)	0.03
Good quality embryos (%)	210/320 (65.6)	37/63 (58.7)	1.38 (0.79–2.38)	0.2
Early cleavage rate (%)	244/320 (76.2)	51/63 (80.9)	0.8 (0.41–1.5)	0.62
Clinical pregnancy (%)	19/83 (22.8)	5/18 (27.7)	0.77 (0.24–2.44)	0.76
Delivery rate (%)	11/83 (13.2)	5/18 (27.7)	0.39 (0.11–1.33)	0.15

CI confidence interval, *MII* metaphase II

Sperm morphology was evaluated with Geimsa staining. Sperm preparation was performed using the swim-up or density gradient techniques as described previously [15]. ICSI procedure was described elsewhere in details [10]. Before the microinjection, evaluation of the maturity and morphology of the oocytes was done using inverted microscope (Nikon TE300, Japan). At the time of ICSI, none of the immature oocytes reached to MII stage. Therefore, they were discarded. Our policy is to microinject only the MII oocytes. The injected oocytes were washed twice, then put in fresh droplets of G1 (Vitrolife co., Sweden) covered with mineral oil (Reploline co., Germany). Degenerated oocytes after microinjection were excluded from the study.

Fertilization and embryo assessments

16–18 h post ICSI, the injected oocytes were checked for presence of the two pronuclei (2PN) and two polar bodies. Fertilization rates were calculated by 2PN no./ MII oocytes no. (%). Unfertilized oocytes were incubated longer and checked again. On day 2, cleaved embryos were evaluated under microscope as reported by [11]. Briefly, embryos were graded as follow: Grade A: equal size blastomeres without fragmentation, Grade B: slightly unequal blastomere, up to 10 % cytoplasmic fragments. Grade C: unequal sized blastomeres up to 50 % fragments and large granules. Grade D: unequal blastomeres with significant fragmentation and large black granules. Grade D embryos were not transferred.

Statistical analysis

Data were shown as mean±S.E. The chi-square and fisher`s exact tests were used for statistical analysis. Independent samples t-test was used wherever appropriate. Also, data were presented as odds ratio (OR), 95 % confidence interval (95 % CI). The ORs refer to the fertilization rate, good quality or early cleaved embryos. Linear (Pearson) correlation test was applied to find the correlation between the number of immature and mature oocytes. P-value <0.05 was considered significant.

Results

The numbers of retrieved immature oocytes were between 0–8 and the average number was 1.26±0.07 per cycle. The mean number of oocytes retrieved and mature ones per cycle was 8.04±0.48 and 6.82±0.45, respectively. The rate of oocyte obtained per follicle was 69.96 %±1.42. The majority of cycles had less than 2 immature oocytes (82.1 %). Also, regarding the number of GV and MI oocytes, 90 % of cases had less than 2 GV oocytes and in 96 % of cases less than 2 MI oocytes were retrieved. Of all the retrieved oocytes, 85 % were mature, while, 9 % and 6 % were at GV and MI stages, respectively.

There was no correlation between the number of immature oocytes and the number of mature ones in each cycle (P=0.99, coefficient of determination (r^2)=0.0000004,

Table 2 Rates of fertilization, formation of good quality embryo, early cleaved embryo, pregnancy and delivery according to the number of GV oocytes

	≤2 GV oocytes	>2 GV oocytes	Odds ratio (95 % CI)	P-value
Number of cases	91	10		
Number of MII oocytes	569	61		
Fertilization rate (%)	346/569 (60.8)	37/61 (60.6)	1 (0.58-1.72)	1
Good quality embryos (%)	228/346 (65.8)	19/37 (51.3)	1.8 (0.91-3.55)	0.1
Early cleavage rate (%)	266/346 (76.8)	29/37 (78.3)	0.89 (0.39-2.03)	1
Clinical pregnancy (%)	21/91 (23)	3/10 (30)	0.7 (0.16-2.94)	0.43
Delivery rate (%)	13/91 (14.2)	3/10 (30)	0.38 (0.08-1.69)	0.19

CI confidence interval, *GV* germinal vesicle, *MII* metaphase II

Table 3 Rates of fertilization, formation of good quality embryo, early cleaved embryo, pregnancy and delivery according to the number of MI oocytes

	≤2 MI oocytes	>2 MI oocytes	Odds ratio (95 % CI)	P-value
Number of cases	97	4		
Number of MII oocytes	596	34		
Fertilization rate (%)	364/596 (61)	19/34 (55.8)	1.2 (0.62-2.49)	0.59
Good quality embryos (%)	235/364 (64.5)	12/19 (63.1)	1.04 (0.4-2.7)	1
Early cleavage rate (%)	284/364 (78)	11/19 (57.8)	2.5 (0.98-6.46)	0.056
Clinical pregnancy (%)	23/97 (23.7)	1/4 (25)	0.93 (0.09-9.4)	1
Delivery rate (%)	15/97 (15.4)	1/4 (25)	0.54 (0.05-5.63)	0.5

CI confidence interval, *MI* metaphase I, *MII* metaphase II

correlation coefficient (r)=−0.00065, CI 95 %=−0.28 to 0.15) (Fig. 1). From total of 320 embryos which were formed in group A, 210 embryos (65.6 %) were good quality embryos and also for cleavage rate, from 320 embryos in group A, 244 embryos were early cleaved. Although, the data showed an increasing trend for formation of good quality embryos in group A compared to group B (65.6 % Vs 58.7 %, respectively) and for early cleavage rate in group B compared to A (80.9 % Vs 76.2 %, respectively), but the difference was insignificant (Table 1).

Out of 509 mature oocytes in group A, 320 oocytes were fertilized (62.8 %) which was significantly higher compared to group B (Table 1). In sub-analysis, no significant differences were recorded between two groups of≤2 and >2 GV oocytes for rates of fertilization (60.8 % and 60.6 %, respectively) and embryo development (Table 2). Also between two groups of≤2 and >2 MI oocytes, fertilization rate and early embryo development were similar (Table 3). Furthermore, clinical pregnancy as well as delivery rate between different groups was insignificant (Tables 1, 2 and 3). The data also showed that there were no significant differences for the maternal age between two groups of A (31.82±0.23) and B (31.08±0.51). Nevertheless, there was significant positive correlation between retrieval of MI oocytes with maternal age (P=0.008).

Table 4 Percentage of normal oocyte and oocyte morphological abnormalities

Criteria	Percentage	Criteria	Percentage
Normal	18.9	Dark	4.1
Fragmented 1 PB	39.7	Irregular ZP	2.4
Refractile body	30.3	Vacuole	1.6
Wide PVS	21.3	Irregular shape	1.4
Granulation	12.2	SERc	1
Debris in PVS	10.6	Small 1 PB	0.6
Central granulation	9	Huge 1 PB	0.5
Bull eye	7		

PB polar body, *PVS* perivitelline space, *ZP* zona pellucida, *SERc* smooth endoplasmic reticulum cluster

The data generated from this study showed that the higher abnormality in oocyte morphology was fragmented 1st pb (Table 4). Likewise, the rate of two oocyte dysmorphisms were higher in women aged over 35, wide PVS and debris in PVS (P=0.004, OR=0.54, 95%CI=0.36-81 and P=0.002, OR=0.42, 95%CI=0.25-.71, respectively). There was insignificant difference for the formation of other oocyte abnormalities (extracytoplasmic and intracytoplasmic) between two groups, except for wide PVS, which was higher in group A (P=0.03, Table 5) (Fig. 2).

Discussion

Many factors may affect the success rate of ICSI such as, maternal age [9], oocyte morphology [16,36], sperm quality [29], ICSI technique [3], injection pipette [30], ICSI operator [28], and quality of transferred embryos [12]. Our data showed that 15 % of retrieved oocytes remained immature. Therefore, to maximize the ICSI success rates, the number and quality of MII eggs in stimulated cycles are important. One of the influential instances maybe the number and the stage of immature oocytes in each cycle. However, the influential effects of immature oocytes on the quality and number of mature oocytes in ICSI are still unclear. Wittemer et al. [34] stated that fertilization rate is higher in IVF cycles with more than 10 % GV oocytes compared to cycles with less GV [34]. They inseminated all cumulus oocyte complexes (including GV, MI and MII) and compared the outcomes. In group with more than 10 % GV, they observed more oocytes with higher fertilization rate. Here, we used number 2 for GV as a cutoff value, and if we used another strategy for choosing cutoff value, we may have found different results. Moreover, Kok and associates showed that the degree of ovarian response and the fraction of immature oocytes may not affect ICSI fertilization rate [18].

Our data demonstrated that the number of immature oocytes may be useful for prognosis of ICSI fertilization outcome. Accordingly, if the number of immature oocytes was higher than two, the fertilization in cohort mature

Table 5 Comparison of different oocyte dysmorphism between two groups of A (≤ 2 immature oocytes) and B (> 2immature oocytes)

	Group A	Group B	Odds ratio (95 % CI)	P		Group A	Group B	Odds ratio (95 % CI)	P
Refractile body	30.45	32.23	1.03 (0.67–1.59)	NS	Dark oocyte	3.73	5.78	0.63 (0.25–1.53)	NS
Central granulation	9.23	8.26	1.12 (0.55–2.3)	NS	SERc	1.17	0.82	1.43 (0.17–12)	NS
Granulation	13.16	8.26	1.68 (0.83–3.37)	NS	Irregular shape	1.17	2.47	0.46 (0.11–1.9)	NS
Wide PVS	22.98	14.04	1.82 (1.05–3.17)	0.03	Vacuole	1.37	2.47	0.54 (0.14–2.15)	NS
Bull eye	7.46	4.95	1.54 (0.63–3.74)	NS	Irregular ZP	2.94	0.82	3.64 (0.47–27)	NS
Debris in PVS	11	9.09	1.23 (0.62–2.43)	NS	Small PB	0.58	0.82	0.17 (0.07–6.9)	NS
Fragmented 1st PB	41.45	32.23	0.67 (0.44-1.02)	NS	Huge PB	0.19	1.65	0.11 (0.01-1.3)	NS

Data are shown as percentage

PB polar body, *PVS* perivitelline space, *ZP* zona pellucida, *SERc* smooth endoplasmic reticulum cluster, *NS* not significant

oocytes was significantly lower. This fact may be related to the possibility that, when immature oocytes are numerous at the pick-up, ovarian follicles may in general be less responsive to the ovarian stimulation. We hypothesize that oocytes collected during cycles with ≤2 immature oocytes have better maturational condition than oocytes retrieval during cycles with more than two immature oocytes. So optimizing the ovarian stimulation protocol to reach this goal is suggested. The data showed that the number of retrieved immature oocytes had no effect on the competence of cohort mature oocytes for good embryo formation. One of the important contributing factor for failed fertilization is the number of retrieved mature oocytes [7]. One probable reason for higher fertilization rate in group A may be due to the increase in the number of mature oocytes available for injection. COH has a key role in ART, and high doses of gonadotropins induce simultaneous growth and development of follicles. One of the causes that retrieved oocytes are in different stages of development is heterogeneity of follicles population at the time of hCG injection [17].

We found that 15 % of oocytes remained immature, which is similar to other report [32]. Some follicles have begun their development in previous cycles, while some are going to develop in this cycle. So, their response to supraphysiologic gonadotropins and their growth speed would be different. It is reported that follicles may have some effects on each other via paracrine secretion. Transforming growth factor-β operates via paracrine or autocrine mechanisms in order to regulate follicular development and oocyte maturation [1,8]. It is mentioned that anti-Mullerian hormone (AMH) inhibits the recruitment of primordial follicles via paracrine activity [6]. Nevertheless, when some oocytes remain immature, in spite of ovarian hyperstimulation, it may be due to the presence of intrinsic defects in the oocytes or even follicles. According to our hypothesis, these immature oocytes may have some negative impacts on the other healthy oocytes via paracrine secretion. Hence, the presence of MI/ GV oocytes after hyperstimulation was not shown to be related with poor outcomes on their mature cohort oocytes.

It is well known that advanced female age is well correlated with the poor quality of eggs. Our data showed that the chance of MI oocyte retrieval is increased in older women. One reason may be related to ovarian function which is decreased with advancing of age as well as the reduction of ovarian response to hyperstimulation. In addition, one of the most important steps in ART is to harvest healthy mature oocytes, which is related to ART success [21]. The findings also showed that the rate of wide PVS is higher in group A, as well as in older women. As mentioned before, we hypothesized that the immature oocytes may have some probable negative effect(s) on the cohort healthy mature oocytes. But, we did not notice any significant differences between rate of oocytes dysmorphism in two groups under investigation, except for wide PVS. Wide PVS may be seen because of overmaturity of cytoplasm at the time of hCG injection. So, PVS enlargement may be related to increased maternal age, and oocyte aging [22,35]. One study detected enlarged PVS as a sign of degeneration/postmaturity in unfertilized human oocytes from an ultrastructural point of view [23]. Also it is suggested that the extracytoplasmic dysmorphisms (e.g. wide PVS) should be considered only a phenotypic heterogeneity of the retrieved oocytes [2]. According to our data, in case of fewer retrieved immature oocytes, the expectation of observing abnormalities which are related to overmaturity may increase. In other words, advanced maternal age and immature oocytes retrieval <2, are two risk factors for these abnormalities. We did not find any significant differences between immature oocyte count and oocyte intracytoplasmic abnormalities. Nevertheless, no significant differences for cytoplasmic abnormalities between young and old women were observed in our population. The causes of cytoplasmic oocyte abnormalities may be multifactorial, for example ovarian stimulation and hormonal environment [27]. Recently, de Cassia et al. [5] showed that the number of retrieved oocytes significantly correlated with increasing incidence of cytoplasmic

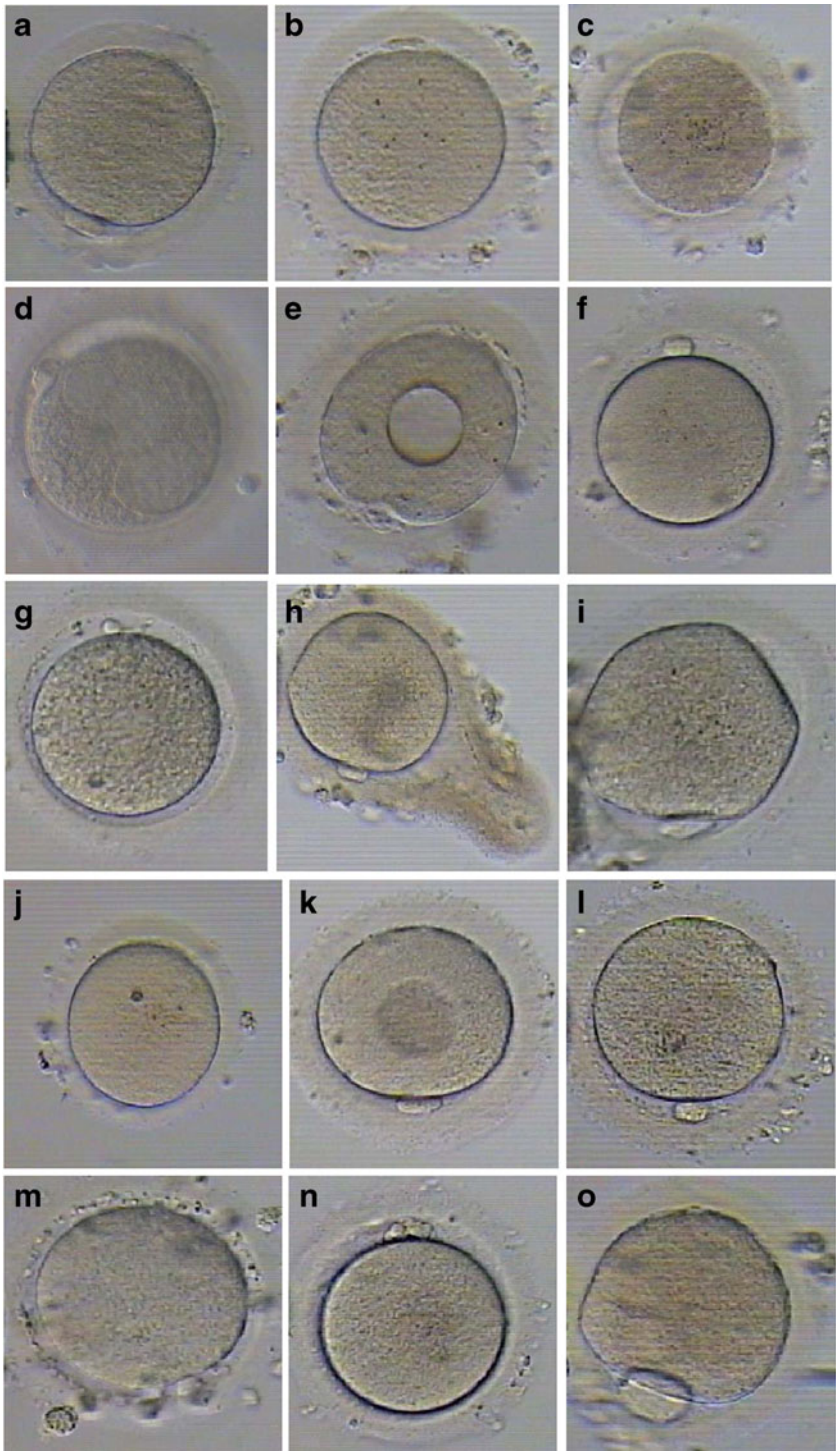

Fig. 2 Different oocyte morphological criteria. **a** normal, **b** refractile body, **c** dark, **d** smooth endoplasmic reticulum cluster, **e** vacuole, **f** wide perivitelline space, **g** small polar body, **h** irregular zona pellucida, **i** irregular shape, **j** bull eye, **k** central granulation, **l** general granulation, **m** debris in periviteline space, **n** fragmented polar body, **o** huge polar body

granularity [5]. They also reported that excessive ovarian response had negative effect on oocyte quality. In the present study, we tried to omit the effect of various ovarian stimulation protocols on oocyte quality, as the patients received long protocol for ovarian stimulation, but various ovarian response and intrinsic variations between the patients may be one reason for seeing these abnormalities. The cause of oocyte morphological abnormalities is probably multifactorial, so larger studies are in need to investigate the relationship between the probable effects of retrieved immature oocytes on feature of mature cohort oocytes.

In conclusion, in cases with few retrieved immature oocytes, the fertilization rates will increase, although it may not have any impacts on early embryo development, or their maturation rate. Besides, advanced maternal age is a risk factor

for harvesting MI oocytes and wide PVS. Although, we did not find any negative impact of immature oocyte count on rates of pregnancy and delivery, but controlled studies with large sample size are necessary to elucidate the role of immature oocytes quantity on ART outcomes.

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