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Embryo Cleavage Rate Is Enhanced By Extending Oocyte-Cumulus Cell Contact: A Randomized Sibling Oocyte Study

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Abstract

Background: Optimal embryonic developmental competence depends on proper oocyte maturation. Nuclear maturation is usually achieved in vivo and can be assessed by the time of ovum pickup; however, cytoplasmic maturation cannot be readily assessed and might be incomplete. Cumulus cells surrounding the oocyte might play a role in the oocyte cytoplasmic maturation.

Objective: To investigate the effect of denudation timing after one and 2 hours on the laboratory and reproductive results in ICSI cycles.

Materials and Methods: In a randomized study including 160 ICSI cycles, 2621 oocytes were randomized into two groups according to denudation timing after 1 or 2 hours of OPU. MII injection was performed immediately. Each patient had 1-2 fresh embryo transfer at day 5.

Results: There is no significant difference in maturation rate between groups I and II. The total fertilization rate between the two groups was comparable at 72.6% & 73.4%, respectively (p=.675). In contrast, the cleaved embryos/ diploid zygotes in group I (94.7%) were significantly lower than those in group II (99.1%, p<.001). Also, cleaved embryos/MII was 68.7% in group I and 72.7% in group II (p=.045). Both groups had no significant difference in the quality of embryos on days 2, 3, and 5. The available blastocysts on day 5 and the transferred grade I blastocysts were comparable. The clinically pregnant females and implantation rates were not significantly different between both groups.

Conclusions: Oocyte-cumulus cell contact is important for cytoplasmic maturity. Although the impact of a higher cleavage rate after 2 hours of preincubation does not affect the clinical pregnancy rate in the fresh cycles, it may increase the probability of achieving a live birth after utilizing all cryopreserved embryos.

Keywords: Oocyte denudation; oocyte- cumulus cells; cytoplasmic maturation; embryo cleavage; ICSI

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Introduction

Intracytoplasmatic sperm injection (ICSI) represents 70 to 80% of the currently performed assisted reproductive techniques (ART) cycles. (1). To improve the success rate of ART, high-quality embryos with high developmental competence should be transferred. Oocyte quality is a critical determinant of embryo developmental competence (2). The oocyte maturation process affects its quality and developmental potential, and both nuclear and cytoplasmic maturation of oocytes are required independently for normal fertilization, which is not guaranteed in stimulated cycles (3). Metaphase II (MII) oocyte that are cytoplasmically immature, are generally associated with poor fertilization rates and reduced developmental potential (4).

For the oocyte to acquire fertilization and developmental competence, the cumulus cells (CCs) surrounding the oocyte play a crucial role in the early stages of maturation, and after oocyte meiosis resumes through both direct cell-to-cell interactions and paracrine factors (5-8). It appears that the timing of maturational events strongly influences the gradual development of oocyte competence, which is only conferred when nuclear and cytoplasmic maturation are tightly integrated (9).

However, cumulus cell removal (denudation; DN) should be performed before ICSI to avoid impedance of the oocyte microinjection by CCs and to enable assessment of the meiotic state of the oocyte, which is limited in the presence of the CCs (10). Generally, there is no set timetable for all ICSI procedures, and the literature input is relatively poor (11). For the oocyte to fully benefit from the somatic cell compartment's presence, it is typical in the embryology laboratory to wait for a certain amount of time—which varies between laboratories—between oocyte pick up (OPU) and DN (12). While most articles agree that preincubation before ICSI is advantageous, the findings about the duration of the preincubation are inconsistent (13). Optimizing oocyte preincubation time can help laboratories plan their workflow effectively and implement corrective steps to improve ICSI results.

There are two leading schools of thought about the timing of denudation. The first encourages a prolonged preincubation period before denudation and immediate injection following denudation (14-15). The second opts for a brief preincubation phase, then denudation and immediate injection (3,16). In the previous studies, immediate oocyte DN and sperm injection (about 36 hours after hCG administration) resulted in low implantation (17), and fertilization rates (18) which were improved with preincubation for at least 2-3 hours (3,16,18). A recent study has reported that oocyte DN within 2 hrs of retrieval is associated with a comparable ICSI cycle outcome compared with oocyte DN between 2 and 5 hrs after retrieval (19), and most studies consider earlier denudation less than 2 hrs as a whole with no randomized studies compared timing within 2 hrs post OPU. Furthermore, a busy IVF/ICSI laboratory may prefer an earlier DN than 2 hours. If the early DN does not compromise the cycle outcome, it may allow more flexibility to the ICSI procedure. Additionally, most of the studies on ICSI timing were retrospective, and few prospective studies focusing on the influence of preincubation time with or without CCs showed conflicting results (13).

Therefore, the study's objective was to prospectively investigate the effect of denudation timing after one and 2 hours on the laboratory and reproductive results in ICSI cycles.

Materials and Methods

Study Design And Population

This trial was designed as a prospective randomized sibling oocyte study. It included 2621 oocytes from one hundred sixty women undergoing IntraCytoplasmic Sperm Injection-Embryo Transfer cycles (ICSI-ET) at the Integrated Fertility Center (IFC) Alexandria between January 2017 and June 2018. The Ethics Committee of the Faculty of Medicine-Alexandria University (17/11/2016) approved the study protocol. Participants' age limit was 38 years, BMI 18-30, had a normal hormonal profile, average ovarian reserve, undergoing ICSI for tubal factor, moderate male factor, or

unexplained infertility. Those with endometriosis or severe male factor requiring surgical extraction were excluded. The oocytes were randomly allocated into one of two groups during OPU. Allocation was not blinded. Oocytes in group I (n=1331) were denuded after one hour of OPU. Oocytes in group II (n=1290) were denuded after two hours of OPU. When there was an uneven number of oocytes, the extra oocyte was assigned to group I because that group's denudation time during the experiment was the norm in our lab. ICSI was started immediately after denudation in each group.

Controlled Ovarian Stimulation and Oocyte Retrieval

All patients had undergone down-regulation for controlled ovarian stimulation (COS) by using a gonadotropin-releasing hormone agonist (GnRHa; Decapeptyl® 0.1 mg, Ferring, Germany) starting on cycle day 21. After down-regulation was confirmed (E2 level <50 pg/ml, with no cysts or follicles >1.0 cm across the maximum diameter), ovarian stimulation was initiated with various gonadotropin preparations containing hMG (Merional® 75mg, IBSA, Switzerland), highly purified hMG (Menopur®; Ferring, Spain) or rFSH (Gonal-F; Serono, Switzerland). The FSH dose was individualized according to the woman's age, BMI, and previous response to ovarian stimulation. Evaluation of the ovarian response and endometrial status by vaginal ultrasound started on the 5th day of stimulation, then every other day to adjust the stimulation dose until three follicles reached ≥18mm diameter, maturation of the oocyte was triggered by IM injection of 10,000 IU hCG (Choriomon® 5000IU, IBSA, Switzerland or Profasi® 5000IU, Serono, Switzerland). The cumulus–oocyte complexes were retrieved under transvaginal ultrasound guidance 35-36 hrs after hCG injection. The cumulus-oocyte complexes were randomly allocated to either group I or group II.

Semen Preparation

Samples of ejaculated semen were collected by masturbation at the time of OPU. The semen was liquefied at room temperature for 30–60 minutes. After evaluating semen parameters according to World Health Organization criteria 2010 (20), the swim-up technique processed the sample (21). Hyperactivated sperms separated from seminal plasma were counted and loaded in the central drop of Quinn's 7% polyvinyl pyrrolidone (PVP) to immobilize the selected sperms. Between 30 min and 2 hours passed between the end of sperm preparation and the start of ICSI.

Denudation and Oocyte Assessment

In each group, oocytes were denuded under stereomicroscope (Olympus SZ2), with high magnification using micropipette with an inner diameter of 0.140–0.150 mm in a two-step procedure, first by pipetting COCs in drops of buffer medium containing human serum albumin HSA and 80 IU/mL of hyaluronidase (SAGE TM), followed by gently pipetting in drops of buffer medium (HTF W/ HEPES, Global life®). The time from oocyte retrieval to the beginning of denudation was recorded. For group I, denudation started after one hour of oocyte retrieval, while in group II, denudation started after 2 hours of oocyte retrieval. Denuded oocytes were transferred to the micro drop of Global® Total media covered with a paraffin oil layer (Ovoil®, Vitrolife, Göteborg, Sweden) in a plastic petri dish. Assessment of the meiotic stage was undertaken by an inverted microscope (Nikon Eclipse Ti-U, Japan). The nuclear status of denuded oocytes was subsequently recorded. Morphologically normal-appearing metaphase II (MII) with a visible first polar body was microinjected. Germinal vesicles (GV) and Metaphase I (MI) degenerated oocytes were not considered for injection in this study. The oocyte maturation rate was defined as the ratio between the MII number and the retrieved oocytes' total number. In each group, microinjection was performed immediately after completion of oocyte denudation. After injection, oocytes were placed in the 40 µl drop of Global® Total covered with oil in the falcon culture dish and further cultivated at standard culture conditions (6.0% Co2, 5.0% O2, and temperature adjusted to 37.0oC) inside a tri-gas incubator (Labotec® C-200, Gottingen, Germany).

Fertilization Check

16–18 hours after ICSI, injected oocytes were examined for fertilization under a 400x inverted microscope (Nikon Eclipse Ti-U, Japan). The presence of two polar bodies and two pronuclei in the oocyte confirms normal fertilization. The ratio between the number of mature injected oocytes and the number of diploid zygotes created was called the fertilization rate (FR).

Embryo Assessment and Transfer

When the fertilized oocyte developed into an embryo with 4-6 blastomeres, it was considered a normal embryonic cleavage. The ratio of cleaved zygotes to the total number of zygotes defines the cleavage rate. Following oocyte retrieval, cleavage-stage embryos in each group were evaluated on days 2 (36–40 hours) and 3 (67–72 hours) based on the number and symmetry of blastomeres and the degree of fragmentation using the British Fertility Society and Association of Clinical Embryologists' cleavage stage grading system (after Cutting et al) (22).

All embryos were moved to 1 mL of medium Gl obal® Total for 48 hours of culture at noon on day 3. On the morning of day 5, embryos were assessed and graded according to the criteria presented by Gardner and Schoolcraft (1999) based on blastocoel expansion and the quality of the inner cell mass (ICM) and trophectoderm (TE) (23). According to the degree of expansion, the blastocysts were scored in ascending order from 1 to 6 (very early, early, expanding, expanded, hatching, or hatched). Beginning with the full blastocyst stage (score 3), an additional assessment of ICM and TE was performed and given a score of two letters (from A to C) based on the number and organization of cells in each. The first index refers to the quality of ICM, and the second index refers to the quality of TE. Then blastocysts were given a grade from I to IV from higher to lower quality to support the entry of scores into numeric databases and facilitate statistical analysis as follows: Grade I: excellent, (≥3AA); Grade II: good, (3,4,5,6, AB and BA); Grade III: average, (3,4,5,6 BB, AC and CA); Grade IV: poor, <3BB. A maximum of two of the best-quality blastocysts obtained were transferred from either group I or group II using a soft transfer catheter (Cook®, Limerick, Ireland) under transabdominal ultrasound guidance. On day 5, surplus grade 3CC or higher blastocysts that had no degeneration were cryopreserved. Patients who needed day 2-3 embryo transfer and those who required mixed day 5 embryo transfer (from group I and group II) were excluded from the study.

Follow Up

Pregnancy was diagnosed by the detection of positive serum β hCG on day 15 after embryo transfer. At 6-7 weeks of pregnancy, a transvaginal ultrasound scan was done to assess implantation, biochemical, and clinical pregnancies (clinical pregnancy was defined by the detection of intrauterine gestational sac with fetal heartbeats) and exclude ectopic pregnancies.

The ratio of the number of gestational sacs to the number of transplanted embryos was used to calculate the implantation rate.

Outcome measures and statistical analysis

At a 5% level of significance and 80% power of the study, a sample size of 170 oocytes, 85 per group, was sufficient to carry out the study. The IBM SPSS software program version 20.0 was used to analyze the data fed into the computer (24). Qualitative data were described in terms of percentage and number (25). The range (minimum and maximum), mean, standard deviation, and median were used to describe quantitative data. The 5% level was used to determine the results' significance. The tests used were the Student t-test, Fisher's Exact, Chi-square, and Wilcoxon signed ranks as appropriate. The number of mature oocytes was the primary outcome that was analyzed, and the rates of fertilization, embryo cleavage, implantation, and clinical pregnancy were the secondary outcomes. Statistics were considered to be significant at a p-value of .05.

Results

To investigate the effect of denudation time on ICSI outcome, our patients could be divided into two groups. Group IP: 92 patients in which embryo transfer was from the oocyte denuded after 1 hour of retrieval, while group IIP: 68 patients in which embryo transfer was from the oocyte denuded after 2 hours of retrieval. Patient demographic data of the two studied groups, including female age, BMI, and cause of infertility, is shown in Table 1. The mean age for the whole study population was 29.5 ± 5.10, with the majority, 132 patients less than 35 years and only 28 above 35 years. The mean BMI value was 25.9 ± 2.90 for the whole study population. Primary infertility represented the majority of patients in group IP (59.8%) and group IIP (70.6%). The most common cause of infertility in both groups was the male factor, which represented 39.1% in group IP and 33.8% in group IIP. There were no significant statistical differences between the two groups.

Table 1: Comparison between the two studied groups according to demographic data.

Demographic data	Total (n=160)		Transfer time				Test of Sig.	*p*-value
			Group IP (n=92)		Group IIP (n=68)			
	No.	%	No.	%	No.	%		
Age (years)								
<35	132	82.5	74	80.4	58	85.3	χ^2=0.639	0.424
≥35	28	17.5	18	19.6	10	14.7		
Mean ± SD.	29.5 ± 5.10		29.46 ± 5.44		29.37 ± 4.64		t=0.109	0.914
BMI (kg/m^2)								
18 – 25	70	43.8	42	45.7	28	41.2	χ^2=0.318	0.573
>25 - 30	90	56.3	50	54.3	40	58.8		
Mean ± SD.	25.9 ± 2.90		25.79 ± 2.84		26.09 ± 3.0		t=0.655	0.514
Cause of infertility								
Primary	103	64.4	55	59.8	48	70.6	χ^2=1.991	0.158
Secondary	57	35.6	37	40.2	20	29.4		
Male factor	59	36.9	36	39.1	23	33.8	χ^2=0.473	0.492
Ovarian	29	18.1	12	13.0	17	25.0	χ^2=3.767	0.052
Tubal	20	12.5	14	15.2	6	8.8	χ^2=1.461	0.227
Uterine	3	1.9	2	2.2	1	1.5	χ^2=0.105	^{FE}p=1.000
Combined	21	13.1	12	13.0	9	13.2	χ^2=0.001	0.972
Unexplained	28	17.5	16	17.4	12	17.6	χ^2=0.002	0.966

χ^2: Chi-square test FE: Fisher Exact t: Student t-test
p: p-value for comparing between the two groups

In total, 2621 oocytes from 160 women were included in this study; 1331 oocytes were denuded after one hour, and 1290 were denuded after two hours. Both groups had a comparable number of mature and immature oocytes at the time of denudation. The maturation rate was 81.7% and 78.7% for groups I and II, respectively. The fertilization rate was also comparable between the studied groups: 72.6% for group I and 73.4% for group II. However, more embryos cleaved from fertilized oocytes denuded at 2 hrs vs. 1 h. Cleavage rate (No. of cleaved embryos /No. of diploid zygotes) was 94.7% & 99.1% for group I and group II respectively, (p <0.001). Also, cleaved embryos/MII was 68.7% in group I and 72.7% in group II (P =0.045). Thus, we only found significant statistical differences between the two groups regarding cleavage rate (Table 2). The total cleaved embryos (day 2 or day 3) were 747 for group I and 738 for group II. However, the number decreased to 442 and 425 blastocysts for groups I and II, respectively, on day 5. Notably, the blastulation rate remained comparable between both groups, 59.2% & 57.6%, respectively, p =0.536. Regarding embryo grading, there were no significant statistical differences between the two groups (Table 2).

Table 2: Laboratory outcome: Comparison between the two studied groups according to the number of denuded oocytes, maturation, fertilization, embryo cleavage, blastulation rates, and day 5 embryo grading.

	Group I	Group II	Test of sig. χ^2	*p*-value
No. of denuded oocytes	**1331**	**1290**		
MII	1087	1015		
Maturation rate (MII /No. of denudated oocytes)	1087/1331 (81.7%)	1015/1290 (78.7%)	3.677	0.055
Fertilization rate (No. of diploid zygotes (2PN)/ No. of MII)	789/1087 (72.6%)	745/1015 (73.4%)	0.176	0.675
Cleavage rate (No. of cleaved embryos /No. of diploid zygotes)	747/789 (94.7%)	738/745 (99.1%)	23.8*	<0.001*
Cleavage rate (No. of cleaved embryos/ No. of MII)	747/1087 (68.7%)	738/1015 (72.7%)	4.026 *	0.045 *
Day 5 embryos	**442**	**425**		
Blastulation rate (blastocyst No. /No. of cleaved embryos)	442/747 (59.2%)	425/738 (57.6%)	0.382	0.536
Day 5 embryo grading				
Grade I	172/442 (38.9%)	171/425 (40.2%)	0.158	0.691
Grade II	94/442 (21.3%)	75/425 (17.6%)	1.809	0.179
Grade III	118/442 (26.7%)	125/425 (29.4%)	0.792	0.374
Grade IV	58/442 (13.1%)	54/425 (12.7%)	0.033	0.855

χ^2: Chi square test *: Statistically significant at $p \leq 0.05$

Positive hCG rate and clinical pregnancy rate were similar in the two groups (Table 3). One hundred sixty embryo transfers were performed, 168 for group IP and 131 for group IIP, with no significant difference in the implantation rate (Table 4).

Discussion

The result of this study indicates that preincubation of oocytes post OPU with intact CC for up to 1 or 2 hours before denudation did not significantly affect oocyte maturation and fertilization rates. However, embryo cleavage rate was significantly higher if oocyte denudation was performed 2 hrs post OPU than 1 hr (72.7% vs. 68.7% respectively, p = 0.045).

However, this didn't significantly affect the blastulation rate. In addition, within this time interval, the most relevant and clinically important IVF endpoints, the implantation and clinical pregnancy rates, did not show any statistically significant differences.

Competent spermatozoa can successfully fertilize only the mature oocytes (26); hence, in ICSI, only MII oocytes were injected after denudation. However, due to the independent acquisition of nuclear and cytoplasmic maturation during folliculogenesis and the possibility of an immature cytoplasm in oocytes retrieved from varying follicle sizes, despite the premature extrusion of the first polar body(1PB), some MII oocytes may not activate and fertilize (27). According to Balakier et al., following 1PB extrusion, human oocyte cytoplasm continues to mature, and ICSI conducted 3-6 hours after the 1PB extrusion increases the fertilization rate (28). In other words, the presence of the 1PB should not be interpreted as a sign of cytoplasm maturity (27). After gaining the ability to undergo nuclear maturation, specific cytoplasmic maturation processes connected to effective preimplantation development most likely continue to take place to allow for the deposition of the maternal components necessary for developing preimplantation embryos.

Although considered ready for ICSI, mature oocytes (with 1PB extruded) may still be in telophase I or prometaphase II. As a result, these oocytes cannot participate in oocyte activation (29), even when fertilization appears normal, developmental defects can subsequently manifest (30).

Table 3: **Clinical outcomes: pregnancy rate**

	Total		Group IP (n=92)		Group IIP (n=68)		χ^2	*p*-value
	No.	%	No.	%	No.	%		
Positive βhCG	85	53.1	48	52.2	37	54.4	0.079	0.779
Clinical pregnancy	81	50.6	46	50.0	35	51.5	0.034	0.854

χ^2: Chi-square test

Table 4: **Clinical outcomes: number of transferred embryos and implantation rate**

	Group IP	Group IIP	χ2	p-value
No. of embryos transferred	168	131		
No. of gestational sacs	60	47		
Implantation rate	60/168 (35.7%)	47/131 (35.9%)	0.001	0.977

χ^2: Chi-square test
Implantation rate is (No. of gestational sacs/ No. of embryos transferred)

Our study found that oocytes' capacity to activate and generate pronuclei did not differ significantly when in vitro culture was extended for up to 2 hours before DN, which may indicate that cytoplasmic maturation was adequate for both groups to sustain this developmental stage. However, significantly more 2PN from group II continues early development and cleavage. This could be attributed to more efficient cytoplasmic maturation achieved with prolonged contact of oocytes with cumulus cells in group II.

Recently, Virant-Klun et al. elucidated the benefit of cumulus cells of mature oocytes in the in vitro maturation (IVM) of immature

oocytes. When immature oocytes are co-cultured, a follicular niche is created to some extent, which helps the immature oocytes mature more quickly and have a gene expression profile that is more similar to that of in-vivo matured oocytes. Additionally, they reported that cleavage rates of the resultant embryos were higher when IVM of cat oocytes were in a co-culture with cumulus cells as opposed to when they were in vitro matured as denuded (10). Fatehi et al. reported that the rate of embryonic cleavage is decreased when cumulus cells are removed from in vitro matured bovine oocytes before IVF. But neither the quantity nor the quality of the resultant blastocysts are impacted. Furthermore, it demonstrates that cumulus cells shield oocytes from hydrogen peroxide (H_2O_2) and that their removal makes oocytes more vulnerable to oxidative stress, negatively impacting subsequent embryonic development. These findings support that cumulus cells guard the oocytes from oxidative damage during fertilization (31).

Ooplasmic dysmaturity has been linked to shorter in-vivo and in-vitro maturation times; therefore, lengthening the time the oocyte interacts with the cumulus complex may help improve ooplasmic maturity (29). Consequently, it can be assumed that the cumulus complex enhances signaling pathways crucial for developing ooplasmic competence (27). By the time of denudation, some of the retrieved metaphase I (MI) oocytes had matured in vitro during preincubation and become ready for sperm injection alongside their in vivo matured sibling oocytes. When fertilized, they have higher rates of cleavage arrest (26). Accordingly, in vitro matured MII oocytes in group I of our study had earlier denudation and thus reduced the chance of adequately completing cytoplasmic maturity (32), which may explain the lower cleavage in this group. Zhang et al. studied cumulus cells' impact on the developmental potential of immature sibling oocytes derived from COS. They found that IVM during ICSI cycles with cumulus cells -intact or denuded oocytes- did not affect nuclear maturation and fertilization, though it improved subsequent embryonic development. Once more, they argued that inadequate oocyte cytoplasmic maturation, not nuclear maturation, is more likely to cause prematurely denuded oocytes' reduced developmental competence (33).

Using the Polscope, we can predict a higher level of embryonic developmental competence by looking for a birefringent meiotic spindle in human oocytes (34). If fertilized, oocytes with missing or malformed spindles have a decreased overall potential to develop into a normal embryo (35). It's possible that they have many chromosomal defects, which could lead to cell cycle arrest (34). Therefore, the delay of injection time in group II of our study allowed more time for meiotic spindle appearance, which agrees with a report by Kilani et al. that meiotic spindle appearance is time-dependent. Spindles were detected higher in MII oocytes at 38–38.5 hrs post-hCG than at 36.5–37.0 hrs post-hCG, with the majority of oocytes having detectable spindles and the highest retardance at 39.0–40.5 hrs after hCG administration, after which they start to disaggregate (36). In line with this, we injected oocytes immediately post DN up to 30 min, corresponding to 36-37hrs ±30 post trigger in group I and 37-38 hrs ±30 post trigger in group II. Thus, group II oocytes belong more to the optimal insemination window proposed by Dozortsev et al, between 37 and 39 hours after hCG treatment, when human oocytes are more likely to become viable embryos. A sort of spindle immaturity might be responsible for the lower cleavage in group I, as some oocytes injected earlier than 37 hours after aspiration at the time of injection result in chromosomal aberrations (17).

Notably, the improvement of early cleavage division in group II has not influenced the rate of blastocyst formation nor the blastocyst quality in this study. Even more, there was no apparent effect on implantation and pregnancy rates, which may indicate that at 1 & 2 hrs retrieval-denudation time, ICSI outcome is comparable. Indeed, the development beyond the eight-cell stage no longer relies on maternal signals; instead, it requires transcriptional activation of the new embryonic genome, which is switched on at about the 4–8-cell stage. Nevertheless, the quality of the developed embryos depends on the intrinsic factors of both gametes involved (37,38). In addition to embryonic development and quality, the

chance of implantation and pregnancy achievement is affected by other significant predictors in an individual patient, including infertility cause, the transferred embryo's chromosomal state, endometrial factors, and sperm quality (39,40).

In agreement with our results regarding the fertilization rate, a recent study by Watanabe et al. retrospectively compared five groups of ICSI cycles where oocytes were immediately incubated in an embryoscope for different periods. The first group included oocytes incubated from 0.5 to 2 hrs, while the other groups included a 1 hr period from 2–6 hrs. They found that the preincubation period is unrelated to fertilization because the actual fertilization rate, as shown by full-time lapse monitoring, was comparable across all groups (41). Our results are also in line with two recent retrospective studies (19,42). Naji et al. recorded the retrieval-denudation times in 2051 consecutive fresh non-donor ICSI cycles with mid-luteal down-regulation or the short GnRH antagonist protocols, and OPU was performed strictly 36 hrs after the hCG injection. They used an automated system that was operator-independent and found that retrieval-denudation times between one and five hours had no effect on fertilization, number of excess embryos at the blastocyst stage, implantation, clinical pregnancy, or live birth rates. (19) Similarly, Barcena et al. analyzed nearly 4000 ICSI cycles and found that various OPU-denudation times and the success of ICSI were not significantly related. They used only young oocyte donors (mean age 26.9 years), a GnRH agonist to trigger oocyte maturation, and primarily performed ICSI with frozen sperm. They combined fresh and frozen embryo transfer (FET) cycles and used cleavage-stage embryo transfer (42).

Although we agreed with Aletebi regarding clinical pregnancy rates, she found higher maturation and fertilization rates when oocyte denudation was performed after 2 hrs rather than 1 hr. However, all the patients studied had undergone COS by the antagonist protocol, and retrieval of oocytes was done approximately 36 hours after hCG administration (18). Previous studies did not show improvement in implantation potential with more extended preincubation periods (3–12hrs) (15,43).

For the best results in fertilization and implantation, Patrat et al. recommended that denudation be attained at least 2 hrs and maybe up to 3 hrs following OPU. ICSI should be carried out as soon as the denudation is completed (16). Compared to our study, this study included a smaller and retrospective sample size. As a result, it is impossible to rule out the risk of bias resulting from variables not considered in the analysis.

We did not find a beneficial effect for nuclear maturation with prolonged oocyte preincubation in vitro before denudation, as we could not get more MII oocytes. In contrast, Ho et al (44). suggested that a preincubation period of ≥ 2.5 hrs in ICSI benefits nuclear maturation. In their study, 64 ICSI cycles were analyzed retrospectively. Patients received long protocol ovarian stimulation, and the injection procedures were performed randomly 1–8 hrs after oocyte retrieval. They found that the maturation rate was increased with a preincubation period of ≥2.5 hrs. Although in our study, we did not extend the preincubation beyond 2.5 hrs, Ho et al. retrieved the oocytes 34 hrs after hCG administration, which may have allowed for observation of lower MII percentages in those oocytes receiving less time for in vivo maturation and in vitro maturation when immediately denuded. Therefore, it was concluded that 34 hrs of in vivo maturation alone is inferior to 34 hrs of in vivo maturation plus at least 2.5 hrs of preincubation in vitro for nuclear maturation of oocytes (44).

The current study also contrasts with previous reports (3,15,16,44), that suggested a pre-denudation time of 2 hrs or more may enhance oocyte maturity, fertilization, embryo quality, or clinical ICSI outcomes. When incubating the oocytes for less than 3 hrs, Rienzi et al showed reduced fertilization rates and embryo morphology in timing groups from 3 to 12 hrs of culture before ICSI (39 to 48 hours after hCG), Although this time would be required for some oocytes to acquire sufficient cytoplasmic maturation and result in a higher activation rate after ICSI, the clinical pregnancy rate analysis

did not reveal any significant differences between the groups, and a higher activation rate upon microinjection may arise from some oocytes needing this time to reach full cytoplasmic maturity, but clinical pregnancy did not differ significantly across groups (15). Our study can't explain this finding as we studied a narrower preincubation period, with the minimum and maximum time that we studied between OPU and ICSI being 1h±30 (36-37 hrs ±30 post-hCG) and 2hrs ±30 (37-38 hrs ±30 post-hCG) in group I and group II, respectively. Isiklar et al have found that ICSI performed right after OPU (36 hours after hCG) resulted in a lower number of MII oocytes, fertilization and cleavage rates, and embryo quality than when at least 2 hrs of incubation before the ICSI procedure (3). However, no difference was found in the implantation or clinical pregnancy rates. Compared to our results, none of the oocytes in our study were randomized to immediate denudation, based on previous reports that also claimed lower implantation rate and fertilization rate with immediate denudation (17- 18).

The strengths of this study are the prospective nature compared with the retrospective design of the majority of available literature regarding timing in ICSI and the average number of ICSI cycles studied, which allows reliable comparisons of cycle outcomes within the study groups. We could account for confounding variables inherent to other trial designs by using randomized sibling oocytes, where each patient acts as their own control. In addition, we intentionally selected a population with an average ICSI outcome, excluding severe causes of infertility, to avoid possible factors that could bias the results.

However, it's crucial to note that because this study only assessed a narrow period of in vitro incubation time, the findings cannot conclusively say if an OPU-denudation time of more than 2 hours could affect the success of ICSI. Furthermore, the timing was recorded manually, several personnel conducted the procedures, and we did not account for cumulative ICSI outcome nor the live birth rate. Only when the results of all subsequent FET cycles are included in the cumulative pregnancy rate or live birth rate can the results of a stimulation cycle be evaluated entirely. One disadvantage of cumulative outcomes is that all FET cycles should be given enough time to complete them.

Conclusion

The present study's most intriguing conclusion is that early embryonic development appears to be enhanced with delayed denudation for two hours post oocyte retrieval, which may further impact cumulative pregnancy rates in day 3 ET-ICSI cycles. However, this difference is overcome by day 5 and does not affect the clinical ICSI outcome, particularly implantation and clinical pregnancy rates.

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