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Effect of Denudation Timing and ICSI on sibling oocytes and Embryo Quality; A Prospective Study

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Abstract

Objectives: To analyses the effects of timing of oocyte denudation and Intracytoplasmic Sperm Injection (ICSI) of sibling oocytes and the effect on Embryo Quality.

Design: We included cases over a two-year period, with the following parameters. Female < 35 years, male factor infertility, antagonist protocol using rFSH for Ovarian Stimulation, the use of freshly ejaculated sperm. (n = 141). Several ICSI parameters were analysed according to the time of denudation and ICSI performed.

The oocytes of each patient were separated into to two groups
Group A, oocytes were denuded at immediately after oocyte pick up and injected immediately.
Group B, oocytes were left two hours and denuded and injected, directly after denudation.

Results: While there was no significant difference in fertilisation rates. The 'Top Embryo Quality' showed a higher percentage in Group 2.

Conclusion: The timing of oocyte denudation didn't show a significant difference in fertilisation rates. Yet waiting two hours for denudation and performing ICSI showed better cell count and grade on Day 3 transfers and better expansion and Inner Cell Mass (ICM) on Day 5 transfers.

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Objectives

The main objective of this study was to see if there was real a significant difference in timing of denudation and ICSI. As a private laboratory our workload varies, from once case a day to eight cases a day. We wanted to see if there really was a difference in top embryo quality based on the timing and denudation of oocytes after retrieval. We used sibling oocytes to make an accurate assessment as possible.

While many genetic tests and time lapse incubators helping us decide which embryo is the most likely to implant and lead to a viable pregnancy. Most of the world especially in my country still heavily rely on Morphological assessment to choose the 'best embryos'.

Introduction

Intracytoplasmic Sperm Injection is treatment of choice for many couples facing infertility. The microinjection technique has been pretty much standardized. Yet there is no standardization of the timing of denudation of ICSI and pick up. Many papers have been published on this topic with varying results. (Rienzi., *et al*. C. Patrat., *et al*.) [1,2].

It has been shown that a pre-incubation period of two hours improves fertilization and pregnancy result; there are some discrepancies in the timing of ICSI. Many studies show that a preincubation period of oocyte and denudation and ICSI improved fertilization rate, embryo quality. (A.O.Trounson., *et al.* K.L.Harrison., *et al*.) [3] While a longer incubation period 9-11 hours was believed to have an adverse effect on embryo quality this probably attributed to oocyte aging. However many other studies showed no statistical differences between fertilization and pregnancy rates? (H.van de Velde., *et al.* M. Jacobs., *et al*.) [5,6].

In theory the differences may attributed to injection time. Oocytes are retrieved before ovulation in the ICSI procedure. According to some reports, pre-ovulatory oocytes are not fully mature despite the presence of a first polar body. This is known as cytoplasmic immaturity (JZ Kubiak., *et al*. H.Balakier., *et al*.) [7,8]. Cytoplasmic maturity is believed to be different to nuclear maturity in artificially induced cycles. Therefore a physically mature oocyte may not necessarily be of optimum fertilizing ability.

Therefore, in theory a pre-incubation period may help the cytoplasmic maturity of the oocyte and hence increase fertilization, and pregnancy rates. This would show that both nuclear and cytoplasmic maturity were needed for optimal results (J.J.Eppigs., *et al*.) [9]. the aim of this study was to compare sibling oocytes on the different timing of denudation and ICSI. We wanted to see if there was a difference in top embryo quality and the optimal timing for denudation and ICSI and follow out corrective measure if possible. Especially in a busy lab.

Materials and Methods

Patients

ICSI was performed at Al Samy Fertility Centre (Mansoura, Egypt) between January 2014 and January 2016. Only were included in this prospective study ICSI with the following parameters. Attempt trial 1 or 2 of ICSI. Female age < 35 years, Male factor infertility, total motile spermatozoa after selection < 1,000,000, At agonist protocol using rFSH for ovarian stimulation. Oocyte retrieval was done 36.5 ± 1-hour after HCG administration [ ] ICSI was done with freshly ejaculated sperm.

Excluded from this prospective study were female patients > 35 years, Less than four oocytes retrieved, more than 15 eggs retrieved. (To avoid cases with Ovarian Hyperstimualtion whose eggs might already have a maturation problem)? We only included patients who used fresh semen sample with a count more than 1 million/ml. We excluded patients using thawed semen or sperm from surgically extracted origin. During the two-year period of this study there were 797 cases in the center, 135 cases were included in this study.

Ovarian Stimulation

Oocyte Preparation

The oocytes were divided into two groups.

Group A, the cumulus and corona cells were removed enzymatically, right after oocyte pick up. ICSI was performed directly after.

Group B, the cumulus and corona cells were removed enzymatically at a later time around an hour or two after incubation. ICSI was preformed directly after.

A total of 494 were injected in Group A.
A total of 176 were injected in Group B.
Only mature MII eggs were injected.

Semen Preparation

Semen samples for the cycles was collected on the morning of oocyte pick up. All samples were obtained by masturbation. After liquefaction, count, motility and morphology was assessed under microscope, according to the World Health Organization Criteria [ ]. Semen was prepared according to the count, motility and morphology. Either swim up or mini swim up or sperm wash. IVF medium chemically balanced salt solution with 0.4% Human Serum Albumin (Ferticult) was used for sperm preparation. The time between sperm preparation and ICSI varied to 30 minutes to 2 hours depending on the workload that day.

ICSI Procedure

Intracytoplasmic sperm injection was done on a micromanipulator microscope. (Narshige) 35 degree angle holding and injection needles were used (RI Research Instruments). All disposable plates and disposables were non-embryo toxic, non-cytotoxic, TC-treated polystyrene, wrapped, sterile plastic-ware (Falcon). Culture media used was non-sequential media (Global) and both Groups were put in the same Incubator 37 degree Celsius, 5% CO_2 (Labotect).

Timing

Different timings were analysed in this prospective study.
The timing for denudation and ICSI done for Group A was done at immediately after oocyte pick-up.
The timing for denudation and ICSI for Group B was done one to two hours after pick-up.
The timing for the cases were recorded before denudation and ICSI.
We attempted to unify times as much as possible for the purpose of this study.

Assessment of Fertilisation

Fertilization for both groups were assessed the day after ICSI. Approximately 17 hours post injection for Group A and Group B.

Fertilization was assessed by presence of second polar body and pronuclear.

Fertilization rate was almost the same in both groups so we assessed the 'TOP' the embryos on Day 3 and Day 5.

The percentage of TOP embryos was the ratio between the number of TOP embryos and total number of mature eggs injected.

There were only two different operators who took part in this gamete and embryo study.

There was no difference between fertilization and implantation rates between the two operators.

TOP Embryo Assessment

Day 3 and Day 5 transfers were done according to the number and quality of embryos present. The TOP embryos that were assessed on Day 3 the number of cells and degree of cell fragmentation. TOP embryos for Day 3 were embryos that had 8 or more cells and were Grade A and B according to the Gardner and Schoolcraft method of embryo classification [iii].

The Day 5 blastocysts were assessed by their degree of Expansion (EXP), Inner Cell Mass (ICM) and Trophectoderm (TE) also using the Gardner and Schoolcraft method of Blastocyst grading. TOP Blastocysts were those with ICM and TE of either A or B. The overall pregnancy rate for the cases was 63.7%.

A specific pregnancy rate for each Group could not be obtained because we would choose the best morphologically sound embryos and in most cases we would take embryos from the two groups on day of transfer.

Objectives

The main aim of this study was to see if different denudation and ICSI timing affected the TOP embryo quality rate. Sibling oocytes were used to gain a better direct comparison. Many papers have been written about this topic with different results. We hoped to gain insight and see if there really was a significant difference and which was better for the embryo morphology and quality. We wanted to use the findings we harvested to help our lab attain the best quality embryos possible and to modify our workflow to our findings.

Results

A total of 135 of the 797 (16.9%) ICSI cycles performed during this study period met inclusion criteria. Mean sperm parameter after selection was 20 x 10^6. A total number of 944 oocytes were received (17+/-5 oocytes per retrieval). 670 eggs were mature (71%) before ICSI procedure. The overall fertilization rate was similar between the two comparative groups. Data was done on embryos considered TOP quality.

Either 8 cell on Day 3 or Blastocyst with Trophectoderm and Inner Cell Mass Quality of C or higher according to the Gardner and Schoolcraft Grading System. 78 oocytes were MI, they represented 10.4% of the total number of oocytes injected. All the MI oocytes were incubated and injected at 11 am to allow for *invitro* maturation.

12 out of the 78 (15.4%) were fertilized. But most of them did not reach TOP embryo quality stage so they were not included in this study. 280 oocytes reached TOP embryo quality out of 670 (41.9%). All cases in this study were given fresh embryo transfer with the average of 1.9 +- 0.6 embryos transferred. The overall pregnancy rate for these selected cases was 63.7%.

A total of 494 were injected in Group A.
A total of 176 were injected in Group B

Statistics

The following tables and charts show the different results of Embryo and their grades.

This table shows the distribution and call grade according to cell count on Day 3 embryo checks for Group A injected at 9 am.

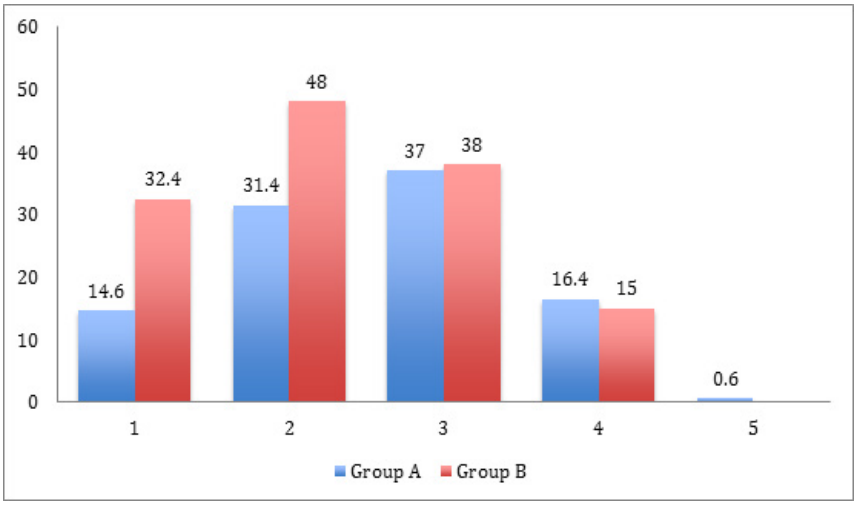

Figure 1: *Comparison between grades for group A and B on D3.*

There was a slight improvement in percentage of Grade 1 and Grade 2 Day3 embryos in Group B injected at 11 am. For the day five transfers the results are as follows in the following tables.

	D5		Significance	Total N = 436
EXP	Group A N = 316	Group B N = 120		
1	26 (8.2%)	8 (6.7%)	P < 0.001	34 (7.8%)
2	65 (20.6%)	25 (20.8%)	P < 0.001	90 (20.6%)
3	101 (32%)	30 (25%)	P < 0.001	131 (30%)
4	106 (33.5%)	29 (24.2 %)	P < 0.001	135 (31%)
5	12 (3.8%)	23 (19.2%)	P < 0.001	35 (8%)
6	6 (1.8%)	5 (4.2%)	P < 0.001	11 (2.5%)

Table 1.1: *Comparison between 9am and 11am Expansion results in Day 5.*

	D5		Significance	Total N = 436
TE	Group A N = 310	Group B N = 120		
A	172 (55.2%)	60 (49.2%)	P < 0.533	232 (53.5%)
B	105 (33.5%)	47 (38.3%)	P < 0.533	152 (34.9%)
C	36 (11.3%)	16 (12.5%)	P < 0.533	52 (11.6%)

Table 1.2: *Comparison between Group A and Group B Trophectoderm results in Day 5.*

	D5		Significance	Total N = 436
ICM	Group A N = 316	Group B N = 120		
A	181 (57.3%)	75 (62.5%)	P = 0.166	256 (58.7%)
B	114 (36.1%)	33 (27.5%)	P = 0.166	147 (33.7%)
C	21 (6.6%)	12 (10%)	P = 0.166	33 (7.6%)

Table 1.3: *Comparison between and Group A and Group B Inner Cell Mass results in Day 5.*

For better clarification we put them bar charts as percentages for each degree of expansion and grade for Trophectoderm and Inner Cell Mass.

It can be seen that there is more expansion in Group A. This is not really significant as it is to be expected given the increase of expansion over time.

The following chart shows the difference in Trophectoderm Grades between the two groups. Group A shows slightly better results for Grade A Trophectoderm than Group B. 55.2% vs 49.2%

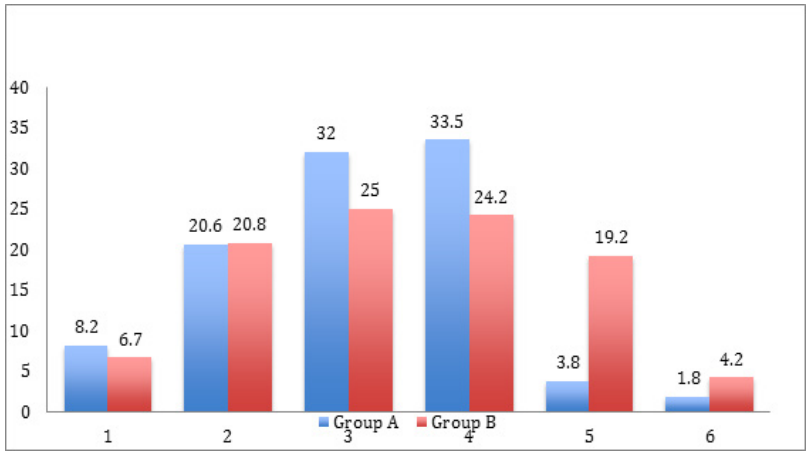

Figure 2: *Comparison between Expansion of Group A and Group B on D5.*

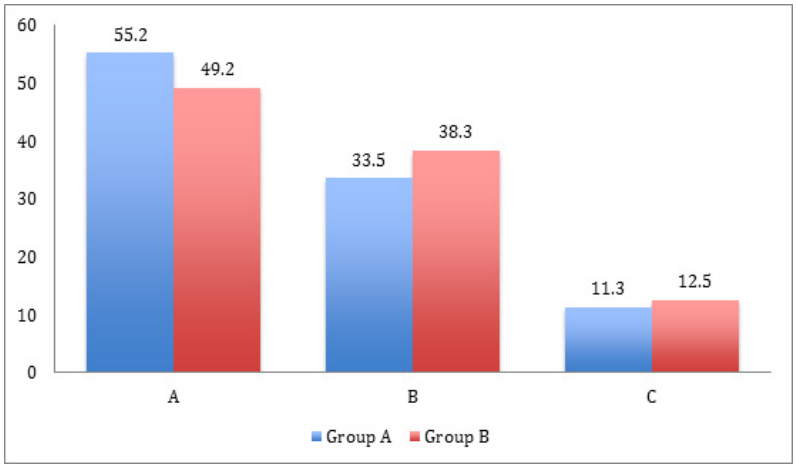

Figure 3: *Comparison between Trophectoderm Grades between Group A and Group B on D5.*

This bar chart shows the difference in percentages and grades between Group A and Group B

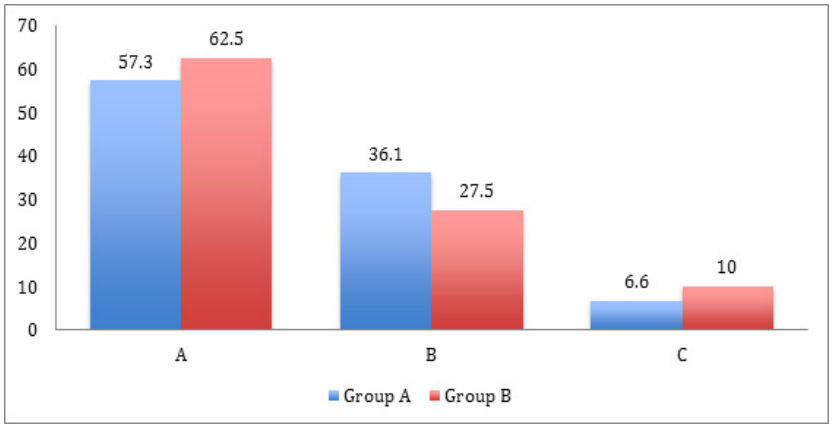

Figure 4: *Comparison between Inner Cell Mass Grades between Group A and Group B on D5.*

There is a higher percentage of Group A ICM mass in Group B. 62.5% vs 57.3% There are many different schools of thought as to which is more detrimental to the overall health of the embryo. The Trophectoderm or the Inner Cell Mass. Data was entered and statistically analysed using the Statistical Package for Social Sciences (SPSS) version 20. Qualitative data was described as means (SD) or medians as appropriate. They were tested for normality by Kolmogorov-Smirnov test.

P value < 0.05 was considered to be statistically significant. P value < 0.01 was considered as high statistically significant.

Discussion

The main objective of this study was to see if there was an effect on embryo quality regarding pre-incubation time and denudation. No significant difference was found between fertilization rates. There was a slight improvement on cell quality for the Day Three transfers for Group B. For the Day Five transfers, there was an improvement of ICM quality in Group B. This alludes to the fact that it might be better to have a waiting period of approximately 60-120 minutes of pre-incubation before denudation and ICSI are performed.

There are many different opinions concerning timing of ICSI and pre-incubation times. However in regards to ICSI only one group has found fertilization rate and quality were reduced with injection occurring hours after oocyte retrieval. The majority of studies showed a slightly significant improvement in embryo quality and pregnancy rates in groups that waited 2-5 hours before denudation and ICSI.

However the quality of embryos seemed to go down when injection was done very late, more than 9 hours. It can be concluded that an optimal time is 2-3 hours before denudation and injection. However since this study only had to comparative groups, it may be beneficial to have further studies with more groups over longer periods of time to detect the best possible timing for denudation and injection.

However still there is no unified opinion of whether or not we should incubate oocytes before denudation and ICSI. There is also no standard protocol for pre-incubation, as there is no common agreement on the amount of times oocytes should be incubated for. For this reason we intentionally selected a specific population to avoid various different factors that could bias results.

However we should not subject pre-incubation times to extremely long hours to avoid pathogenic activation. It has been shown that oocytes incubated for very long times 9-11 hours post retrieval, show poorer quality (K.Yanagida., *et al.* M.Plachot., *et al.* J. Tesarik., *et al*.) [14-16]. The ability of the oocyte cytoplasm to of MII oocytes to decompensate sperm DNA, complete meiosis and promote the male pronucleus appears to decrease the longer ICSI is done after oocyte retrieval.

Some authors Van de Velde [5] claimed that ICSI should be done no later than 12 hours post retrieval due to invitro aging and meiotic spindle instability and chromosome scattering in the oocyte. One study even claimed the only benefit of pre-incubation before injection was the increase of MII eggs we obtained (Ho., *et al.*) [13]. The better quality and rate of TOP embryos in Group B maybe due to the fact that pre-incubation allows more time for cytoplasmic maturity [1].

We know if an oocyte is mature or not by the presence of an extruded polar body. Cytoplasmic maturity is believed to involve proteins and mRNA. In natural unstimulated cycles nuclear and cytoplasmic maturity are synchronous. However in stimulated cycles, oocytes maybe physically mature with an extruded polar body yet cytoplasmic ally immature. This could be a reason why there is a better quality embryo in Group B. The granulose and cumulus cells are essential for oocyte nuclear and cytoplasmic development. The surrounding cells might also secrete paracrine and growth factors, which may play a role in the nuclear and oocyte maturation.

Conclusion

Our studies show that there is a slight difference in TOP embryo quality between the two groups. An incubation time of approximately 60-120 minutes has shown to have some benefit. This is good to know in a busy laboratory as pre-incubation times 2-5 hours show no detrimental effect on Embryo Quality.

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