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Impact of the Volume of Cytoplasm Aspirated into the Injection Pipette at the Time of Oolemma Breakage on the Fertilization Rate after ICSI: A Preliminary Study

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Abstract: *Purpose: To investigate the impact of the volume of cytoplasm aspirated into the injection pipette on the fertilization, embryo development and implantation ability after intracytoplasmic sperm injection. Methods: This was a preliminary observational study conducted between October 2010 and December 2010. We divided oocytes into two groups based on the farthest point reached by the aspirated cytoplasm. The intersection of the ICSI pipette and the outer surface of the zona pellucida was used as a marker. When the farthest point reached by aspirated cytoplasm from the tip of the injection pipette was less than the marker, we classified the oocyte as group A, and when it was beyond the marker, we classified the oocyte as group B. Results: The oocyte degeneration rate of group A (7%) was higher than that of group B (3%), but the difference was not significant. The fertilization rate (91% versus 71%) was significantly higher in group A than in group B (P = 0.007). On the other hand, the rates of survival, cleavage, good quality day-3 embryo, good quality blastocyst and pregnancy following single day-3 embryo or day-5 embryo transfers were similar between the two groups. Conclusions: These results indicate that the volume of cytoplasm aspirated into the injection pipette affects the fertilization rate, but does not influence embryo development or implantation ability after intracytoplasmic sperm injection.*

Key words: *Cytoplasm, Fertilization, Human, Intracytoplasmic sperm injection, Volume*

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Introduction

Intracytoplasmic sperm injection (ICSI) is a method of *in vitro* fertilization (IVF) in which a single sperm is introduced directly into the cytoplasm of a mature oocyte, and has rapidly become the preferred treatment for male factor infertility, since its first reported pregnancies and births in 1992 [1]. In addition to male factor fertility treatment, the use of ICSI has expanded to a variety of other applications, such as fertility treatments after *in vitro* insemination failure, or using cryopreserved sperm. Some researchers have even advocated the generalized use of ICSI for all patients undergoing IVF [2].

ICSI is performed by the mechanical penetration of the zona pellucida and oolemma by a glass needle through which a sperm is injected into the cytoplasm. In order to inject the sperm into the oocyte, oolemma breakage is essential. Aspiration of cytoplasm into the injection pipette has been shown to be necessary for breakage of the oolemma [3]. There are several ways to break the oolemma. At our clinic, we connect a pneumatic injector to the injection pipette and aspirate air. The cytoplasm is aspirated slowly into the pipette, then a sudden flow of cytoplasm into the pipette occurs. We consider this moment of sudden flow defines the oolemma breakage, and stop the aspiration of air, to stop the cytoplasm flow. We, then deliver the sperm together with the aspirated cytoplasm back into the oocyte. Although, we aspirate the same volume of air, the farthest point reached by aspirated cytoplasm in the pipette at the time of oolemma breakage varies for each oocyte and little information is available about the impact of the volume of cytoplasm aspirated into the injection pipette at the time of oolemma breakage on the fertilization, embryo development and

implantation ability after intracytoplasmic sperm injection. Dumoulin *et al.* reported that the volume of cytoplasm aspirated during the injection procedure does not have an impact on the fertilization rate, but the zygotes resulting from a relatively large volume of cytoplasm aspiration, have a lower potential to develop into blastocysts [4]. In the present study, the rates of fertilization, survival, cleavage, embryo quality, good quality blastocyst formation and pregnancy rate of day-3 and day-5 embryo transfers after ICSI were examined with respect to the volume of cytoplasm aspirated into the injection pipette at the time of oolemma breakage.

Materials and Methods

We retrospectively investigated 132 microinjected mature oocytes (mean number of mature oocytes ±SD per woman=5.1 ± 3.8) which were selected from 153 oocytes (mean number of oocytes ±SD per woman=5.9 ± 4.5) retrieved from 26 patients (mean age of women ±SD=37.3 ± 4.7 yr) between October 2010 and December 2010. Indications for ICSI were diagnosis of moderate to severe male factor infertility or a history of total fertilization failure in previous IVF with conventional insemination. All patients undergoing IVF with ICSI at NIJI Clinic during the study period were eligible to participate in the study which was limited to one cycle per patient. The only exclusion criterion was retrieval of less than one mature oocyte. We used a commercially available micro-injection pipette (K-MPIP-1035, Cook Ireland Ltd., Ireland). The inside diameter of injection pipette was 5 μm and outside diameter was 7 μm. The injection pipette was connected to a pneumatic injector (IM-9C, NARISHIGE Inc., Japan). One rotation of knob was approximately 480 μl. The preparation of the injection pipette was done as follows. First, we aspirated Hepes-buffered medium (SYDNEY IVF GAMETE BUFFER, Cook Australia Pty Ltd., Australia) into the injection pipette by capillary phenomenon for 1 min, then we aspirated 7% polyvinylpyrrolidone (7% PVP Solution, Irvine Scientific, USA) by aspirating 160 μl of air for 5 min. A motile sperm was immobilized by touching the tail with the injection pipette, and aspirated tail first into the injection pipette with in a PVP (Irvine) solution drop. The injection pipette was inserted through the zona pellucida well into the oocyte (~80% of the oocyte diameter), with the polar body at 12 o'clock (Fig. 1a, b). At the time of ICSI, we assessed the morphology of the first polar body as intact or fragmented and checked for the presence of cytoplasm vacuoles [5, 6]. The oolemma breakage procedure was performed as follows. Air, 400 μl, was rapidly aspirated using a pneumatic injector connected to the injection pipette. Cytoplasm is at first slowly aspirated into the injection pipette; then, a sudden flow of cytoplasm into the injection pipette occurrs. We consider this moment to be the moment of oolemma breakage and discharge the 400 μl of air as quickly as possible to stop the cytoplasm flowing into the injection pipette. During the procedure, the farthest point reached by the aspirated cytoplasm in the glass needle was observed. As we had no recording devices, such as camera, monitor or video recorder connected to the microscope during the period of study, we defined the intersection of the ICSI pipette and the outer surface of the zona pellucida as a marker (Fig. 1a, b: red line) for dividing the oocytes into two groups based on the volume of cytoplasm aspirated into the injection pipette (Fig. 1a, b: yellow bar): group A, the farthest point reached by the

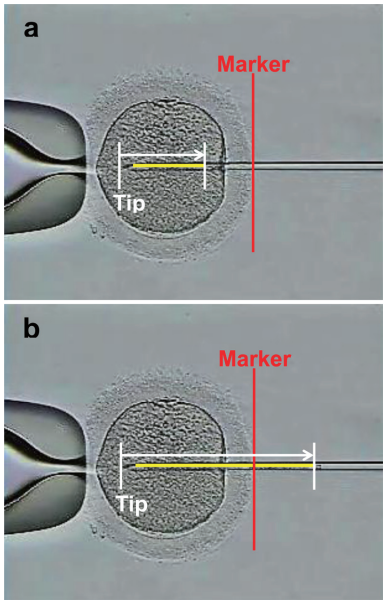

Fig. 1. We defined the intersection of the ICSI pipette with the outer surface of the zona pellucida as a marker (a, b: red line) for grouping the volume of cytoplasm aspirated into the injection pipette (a, b: yellow bar) into two groups. Group A: the farthest point reached by the aspirated cytoplasm from the tip of the injection pipette did not reach the marker (a). Group B: the farthest point reached by the aspirated cytoplasm from the tip of the pipette was beyond the marker (b).

aspirated cytoplasm from the tip of the injection pipette was less than the marker (Fig. 1a); and group B, the farthest point reached by the aspirated cytoplasm from the tip of the pipette was beyond the marker (Fig. 1b). There were 100 oocytes in group A and 32 oocytes in group B. After oolemma breakage, we introduced 40 µl of air into the injection pipette using the pneumatic injector to inject the sperm into the oocyte. After the period of study, we measured the exact farthest point reached by the aspirated cytoplasm relative to the tip of the pipette, as well as the pipette dimensions (inner diameters at the tip of the pipette and at the farthest point reached by the aspirated cytoplasm) on a monitor screen. The farthest point reached by the aspirated cytoplasm was converted to volume (in pl) by calibrating the monitor measurement using a micrometer scale under the microscope at the same magnification. We measured 10 oocytes in group A and 10 oocytes in group B in order to investigate the correlation between the volumes of cytoplasm aspirated into the injection pipette of group A or B and the marker. Each ICSI procedure was performed by only one embryologist in order to minimize the possibility of technical-related factors influencing the results.

Embryo culture and assessment

The day of oocyte retrieval was considered as day 0. After ICSI, oocytes were cultured individually in 15µl droplets of cleavage medium (Cook Australia Pty Ltd., Australia) covered by mineral oil (OVOIL, Vitrolife, Sweden) from day 1 to day 3. Fertilization was confirmed at 24–25 h after oocyte retrieval (day 1) by the presence of two pronuclei. All oocytes and embryos were incubated at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2. On day 3, embryos originating from normally fertilized zygotes were observed. Good quality embryos were defined as those having regular blastomeres, <20% fragments and those containing at least seven cells. On day 3, either a single good quality embryo was transferred, or 1–3 good quality embryos were cryopreserved on day 3 and surplus embryos were transferred and cultured individually in 15µl droplets of blastocyst medium (Cook) covered by mineral oil (Vitrolife) from day 3 to day 5. On day 5, embryos were examined for development into blastocysts. Good quality blastocysts were defined as those scoring B or higher for both inner cell mass and trophectoderm grades (i.e. BB) [7]. After single good quality blastocyst transfer on day 5, surplus good quality blastocysts were cryopreserved. Fresh embryo or blastocyst transfer was cancelled for some patients in order to avoid potential risks arising from ovarian hyperstimulation syndrome or because of thin (<8 mm) endometrium at the time of oocyte retrieval. The embryos and blastocysts were vitrified by the method developed by Kuwayama *et al.* [8] using a cryotop (Kitazato Supply Co., Fujinomiya, Japan), albeit with slight modifications; the method was described previously [9, 10]. Single vitrified-warmed embryo or blastocyst transfers were performed in spontaneous cycles or in hormonal replace treatment cycles.

Statistical analysis

The Mann-Whitney U-test, the unpaired *t*-test, the Chi-squared test and Fisher's exact test were used as appropriate to determine statistical differences between groups. A P value of <0.05 was considered significant.

Results

There were no significant differences between the groups in the mean age of the women, mean number of IVF attempts, previous survival rates and fertilization rates of ICSI in the previous IVF treatment (Table 1). At the time of ICSI, we assessed the morphology of the first polar body as normal or fragmented and checked for the presence of cytoplasm vacuoles. There were no significant differences in the percentages of oocytes with normal or fragmented morphology and the percentages of oocytes with or without vacuoles between the two groups (Table 2). The oocyte degeneration rate of group A (7%; 7/100) was higher than that of group B (3%; 1/31), but the difference was not significant. The fertilization rate was significantly higher in group A than in group B (91%; 85/93 versus 71%; 22/31) ($P = 0.007$). On the other hand, the rates of survival, cleavage, good quality day-3 embryo, good quality blastocyst and pregnancy following single day-3 or day-5 embryo transfers were similar between the two groups (Table 3). The volume of cytoplasm aspirated into the injection pipette (2.5 ± 0.8 pl versus 3.9 ± 0.2 pl; mean ±SD) was significantly more in group A than in group B ($P = 0.0002$) (Table 4).

Discussion

The results of the present study indicate that the volume of cytoplasm aspirated into the injection pipette at the time of oolemma breakage affects the fertilization rate, and larger volumes of aspirated cytoplasm were associated with lower fertilization rates. However, the oocyte degeneration rate, the survival rate, the embryo development and the pregnancy rate were not influenced by the volume of cytoplasm aspirated into the injection pipette.

Table 1. Characteristics of patients and previous fertilization rate of ICSI

	Group A	Group B	*P* value
No. of patients	23*	17*	
Mean age of women ± SD**	37.2 ± 5.0	37.1 ± 4.0	NS
Mean no. of IVF attempts ± SD***	3.5 ± 3.4	2.8 ± 2.2	NS
Previous survival rate of ICSI****	89%	90%	NS
Previous fertilization rate of ICSI****	63%	66%	NS

*14 patients overlapped, **Student's t-test, *** Mann-Whitney U-test, ****Chi-squared test, NS = not significant.

Table 2. Characteristics of oocytes at the time of ICSI

	Group A	Group B	*P* value
No. of oocytes	100	32	
First polar body (PB) morphology			
No. of oocytes with normal PB (%)*	73 (73)	25 (78)	NS
No. of oocytes with fragmented PB (%)*	27 (27)	7 (22)	NS
Presence of cytoplasm vacuoles			
No. of oocytes without vacuoles (%)*	95 (95)	31 (97)	NS
No. of oocytes with vacuoles (%)*	5 (5)	1 (3)	NS

*Fisher's exact test, NS = not significant.

Table 3. Effect of the volume of cytoplasm aspirated into the injection pipette on fertilization, embryo development and pregnancy rates

	Group A	Group B	*P* value
No. of oocytes receiving ICSI	100	32	
No. of oocytes degenerating after ICSI (%)*	7 (7)	1 (3)	NS
No. of oocytes surviving after ICSI (%)*	93 (93)	31 (97)	NS
No. of oocytes fertilized (%)*	85 (91)	22 (71)	0.007
No. of embryos cleaved (%)*	83 (98)	22 (100)	NS
No. of good quality day 3 embryos (%)**	53 (64)	13 (59)	NS
No. of embryos cultured to the blastocyst	60	15	
No. of good quality blastocysts (%)*	22 (37)	5 (33)	NS
No. of single day 3 embryo transfers	10	5	
No. of clinical pregnancies after single day 3 embryo transfers (%)*	4 (40)	2 (40)	NS
No. of single day 5 embryo transfers	5	4	
No. of clinical pregnancies after single day 5 embryo transfers (%)*	3 (60)	2 (50)	NS

*Fisher's exact test, **Chi-squared test, NS = not significant.

Table 4. Volume of cytoplasm aspirated into the injection pipette

	Group A	Group B	*P* value
No. of oocytes	10	10	
Mean volume of cytoplasm aspirated into injection pipette ± SD* (pl)	2.5 ± 0.8	3.9 ± 0.2	0.0002

*Mann-Whitney U-test.

Although the difference was not significant, the oocyte degeneration rate of group A (7%) was higher than that of group B (3%). The volume of cytoplasm aspirated into the injection pipette is associated with the timing of oolemma breakage. As the same volume of air was aspirated by the pneumatic injector connected to the injection pipette for all oocytes, the oolemma breakage in group A oocytes occurred faster than that in group B oocytes. In

other words, the tension of the oolemma of the oocytes in group A was low and that of the oocytes in group B was high. There were no significant differences between the groups in the mean ages of the women, mean number of IVF attempts, previous survival rates and fertilization rates of ICSI in the previous IVF treatment. In addition, there were no significant differences in the percentages of oocytes with normal or fragmented morphology and the percentages of oocytes with or without vacuoles between the two groups, suggesting that the qualities of the oocytes were similar between the two groups. Additionally, as 19 patients had both group A and B oocytes, the tension of the oolemma varied among oocytes, not among individuals. In group A oocytes, in which faster breakage of the oolemma occurred, the oocyte degeneration rate was higher than in group B oocytes, in which the oolemma broke slowly. Palermo *et al.* found significantly higher damage rate (13.9%) in the sudden oolemma breakage group compared to the normal and difficult oolemma breakage groups (4.3% and 2.9%) [11]. Similar results were obtained by others [4, 12, 13], suggesting that the timing of oolemma breakage has an effect on the oocyte degeneration rate after ICSI.

Our results demonstrate that the volume of cytoplasm aspirated into the injection pipette had an impact on the fertilization rate but not on the blastocyst rate. These results are not in agreement with those reported by Dumoulin *et al.* [4], who reported that the volume of cytoplasm aspirated into the injection pipette (≤2, 2–3, 3–4, 4–5, 5–6, >6 pl) did not influence the fertilization rate, but that the blastocyst rate was compromised in a group of embryos originating from oocytes in which >6 pl of cytoplasm was aspirated into the injection pipette. It could be postulated that when >6 pl of cytoplasm are aspirated into the injection pipette, the large volume of aspirated oolemma might obstruct the opened hole at the time of sperm injection. However, the aspirated oolemma was removed by injecting a greater volume of PVP solution, which might have helped the sperm injection but might also have compromised the blastocyst rate, probably due to sublethal damage inflicted on the oocyte. On the other hand, in our study, it could be postulated that the greater the volume of cytoplasm aspirated into the injection pipette, the greater the volume of oolemma aspirated, which might have obstructed the opened hole when the sperm was injected together with all of the cytoplasm back into the oocyte. However, the volume of PVP solution injected into the oocyte was constant irrespective of the volume of cytoplasm aspirated into the injection pipette, because we exerted the same pressure on the injection pipette at the time of sperm injection. Therefore, the embryo development and implantation ability could not have been influenced by the volume of cytoplasm aspirated into the injection pipette.

A limitation of our study is that the marker (intersection of the ICSI pipette and the outer surface of the zona pellucida; Fig. 1a, b: red line) used in the present study to divide the oocytes into two groups based on the volume of cytoplasm aspirated into the injection pipette was not strict, because the thickness of the zona pellucida, the perivitelline space and the diameter of each oocyte varies. However, this marker is simple, easy to see and may be used as an indicator when assessing the ICSI procedure, especially at the time of oolemma breakage. In addition, we found a significant correlation between the volumes of cytoplasm aspirated into the injection pipette of groups A and B and the marker. Nevertheless, the population of this study was small and this was a preliminary study, so further studies will be needed.

In conclusion, we suggest that one way to improve the fertilization rate of ICSI, is to reduce the volume of cytoplasm aspirated into the injection pipette at the time oolemma breakage. Kimura and Yanagimachi reported that a method of piezo-ICSI for mice oocytes, in which there was no need to aspirate the cytoplasm into the injection pipette at the time of oolemma breakage [14]. Yanagida *et al.* applied piezo-ICSI to human ICSI and reported a significantly higher fertilization rate using piezo-ICSI in comparison with conventional ICSI [15]. The elimination of aspiration of cytoplasm into the injection pipette at the time of oolemma breakage would improve the fertilization rate of ICSI, and studies are in progress to clarify whether piezo-ICSI can improve the results of ICSI in comparison with conventional ICSI.

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