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Comparison of fertilization outcome between microdrop and open insemination methods in non-male factor IVF patients

Yubin Li, Tao Li*, Qingyun Mai, Lingli Long, and Jianping Ou

The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

Abstract

Both microdrop and open methods are commonly used for *in vitro* fertilization (IVF) protocols for embryo culture as well as oocyte insemination. However, few comparative studies evaluating the microdrop or open method of insemination on the fertilization outcome and subsequent embryo development have been performed. A randomized study was conducted to compare microdrop and open fertilization with respect to fertilization rate and embryo development among non-male factor patients undergoing *in vitro* fertilization and embryo transfer (IVF-ET). The results presented in this study demonstrate that the fertilization failure rate [total fertilization failure rate (TFF) plus low fertilization rate (<25% oocytes fertilized)] in the microdrop insemination group was higher than in the open insemination group (11.9% versus 3.3%, $p < 0.001$), while the good quality embryo rate and pregnancy rate did not differ significantly between the groups. As a highly complicated process involving many extrinsic and intrinsic factors, further studies are needed to confirm the effects of these insemination methods on the rate of fertilization failure.

Abbreviations: IVF-ET: *in vitro* fertilization and embryo transfer; TFF: total fertilization rate; GnRHa: gonadotropin-releasing hormone analogue; rFSH: recombinant follicle stimulating hormone; PN: pronuclei

Keywords

Fertilization failure, *in vitro* fertilization, insemination, sperm

History

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Introduction

In vitro fertilization (IVF) involves co-incubation of oocytes with the appropriate number of sperm in an easily controllable and physiologically amenable environment [Green 1993]. Currently, varying volumes of insemination media and various culture vessels are used for oocyte fertilization and a variety of insemination methods are used in clinical IVF [Dai et al. 2012; Marrs et al. 1984; Söderlund and Lundin 2006]. Strategies include 4–5 well dishes [Marianowski et al. 2007] or organ culture center well dishes [Suh et al. 2006], and microdrop insemination methods categorized as with oil overlay or cover, and open insemination methods without oil overlay.

Here we focused on 'two common' approaches, microdrop and open insemination methods. Microdrop insemination is achieved by preparing 50–100 µl droplets of sperm suspension that is covered with liquid oil, and incubating oocytes in those sperm droplets for fertilization. In comparison, the open insemination method consists of a measured volume of prepared sperm suspension that is directly added to each well containing oocytes without oil overlay for fertilization [Gianaroli et al. 1996]. Both the microdrop and open insemination method is employed in the IVF laboratories today. The choice of which method is employed in IVF laboratory is by practice, but most importantly IVF-ET outcome.

Few comparative studies have been performed to evaluate the microdrop or open methods, on fertilization outcomes and subsequent embryo development. One study showed that the fertilization rate and the fertilization failure rate between the microdrop and the open insemination methods were not significantly different [Boone and Johnson 1997]. Nevertheless, our retrospective data analysis had associated a higher fertilization failure rate with the microdrop insemination method as compared to the open method. Moreover, mineral oil used for microdrop insemination methods had been indicated to be a source of toxic contaminants for fertilization and embryo culture [Morbeck and Leonard 2012; Otsuki et al. 2007; Sifer et al. 2009].

To determine whether these insemination methods affected fertilization outcome, we prospectively compared the fertilization failure rate, and embryo development between microdrop and open fertilization methods in a large number of conventional non-male factor IVF patients. The relative efficiency of each insemination method was assessed by the overall fertilization rate, the fertilization failure rate,

*Address correspondence to Tao Li, PhD, The Reproductive Center, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China, Tel: +86-20-87755766-8362/Mobile: +86-13725342880, E-mail: lilitao@gmail.com

Table 1. Demographic data of the women in the microdrop insemination and the open insemination groups.

	Microdrop insemination group	Open insemination group	*P*
Case (*n*)	573	602	–
Female age, mean ± SD (years)	31.8 ± 4.1	32.1 ± 3.7	NS
Body mass index, mean ± SD (kg/m^2)	21.3 ± 2.1	21.7 ± 1.8	NS
Infertility diagnosis, *n* (%)			
Tubal factor	306 (53.4)	300 (49.8)	NS
Endometriosis	30 (5.2)	28 (4.7)	NS
Unexplained	184 (32.1)	226 (37.5)	NS
Anovulation	53 (9.2)	48 (8.0)	NS
Quantity of gonadotrophins used, mean ± SD (IU)	1998.5 ± 246.4	2023.7 ± 291.1	NS
No. of stimulation days, mean ± SD	10.5 ± 2.2	10.4 ± 3.0	NS
No. of oocytes retrieved, mean ± SD	12.0 ± 4.9	12.4 ± 6.1	NS
No. of mature oocytes, mean ± SD	10.1 ± 3.2	9.7 ± 2.8	NS

NS: not significant

Table 2. Descriptive statistics of semen specimens provided for the *in vitro* fertilization.

Parameters	Microdrop insemination	Open insemination	*P*
Pre wash (semen)			
Sperm concentration, mean ± SD ($\times 10^6$/ml)	73.2 ± 27.3	72.6 ± 30.1	NS
Sperm progressive motility, mean ± SD (%)	62.2 ± 13.6	59.8 ± 16.5	NS
Percentage of normal morphology* (%)	6.18 ± 2.9	5.74 ± 3.6	NS
Post swim-up (sperm suspension after sperm selection)			
Sperm concentration mean ± SD ($\times$106/ml)	47.3 ± 10.4	46.9 ± 14.1	NS
Sperm progressive motility, mean ± SD (%)	92.4 ± 9.7	93.1 ± 10.5	NS
Final concentration for insemination, mean ± SD	0.3×10^6/ml	0.3×10^6/ml	NS

*Based on WHO-5 standard.

and abnormal fertilization rate. Embryo development was compared between the two methods as reflected by the cleavage rate, the rate in which good quality embryos were obtained, and the pregnancy rate.

Results and Discussion

Demographic data and sperm parameters in the two groups

The data in Table 1 indicated that all relevant demographics including female age, infertility diagnosis, BMI, quantity of gonadotrophins used, stimulation days, number of oocytes retrieved, and number of mature oocytes were similar for the microdrop and open insemination groups ($p > 0.05$). Because with preconditions, such as PCOS, POF, and autoimmune diseases, can affect oocyte quality and thus the fertilization rate, we excluded these patients from our study [Kilic et al. 2008; Zhang et al. 2013]. The patients undergoing treatment with hormones within three months of the study were also excluded [Shastri et al. 2011], as the effects of hormones on outcomes measured in this study remain controversial [Decanter et al. 2013]. The fertilization rate was a function of the rate of mature oocytes. Accordingly the fertilization failure rate was only considered as a function of mature oocytes.

Moreover, sperm parameters (concentration and progressive motility) of semen and sperm suspension after selection were not different between the two groups, as shown in Table 2. The concentration of sperm used for insemination is essential for normal and stable normal fertilization rates [Chiamchanya et al. 2008]. The concentration of sperm used for insemination has deceased dramatically within the last 10 years. The final concentration of sperm used for insemination within this study was 0.3×10^6/ml.

Insemination methods and oocyte fertilization

In total, 14,360 oocytes from 1,175 women were included in the study. The microdrop insemination group included the analysis of 6,887 oocytes from 573 women, and the open insemination group included the analysis of 7,473 oocytes from 602 women. Notably, 28 women had total fertilization failure (TFF) and 40 patients had a low fertilization rate (defined as <25% of oocytes fertilized) in the microdrop insemination group (total 68/573, 11.9%). In comparison only seven women had TFF and 13 women had a low fertilization rate in the open insemination group (total 20/602, 3.3%). Thus, the fertilization failure rate was significantly higher in the microdrop insemination group as compared to that in the open insemination group (11.9% versus 3.3%, $p < 0.001$) (Table 3).

The reported fertilization failure rate [total fertilization failure plus low fertilization rate (defined as <25% fertilization)] within a normospermia group varies from 5% to 25% of couples undergoing conventional IVF [Allgeyer et al. 2006; Chiamchanya et al. 2008]. This might partly be due to the different sample sizes and some common factors. For example, sperm morphology, the acrosome reaction, and the interaction between the sperm and the zona pellucida, have been shown to be associated with sperm penetration and fertilization failure [Allgeyer et al. 2006; Liu and Baker 2000; Zhu et al. 2013]. But there are factors that cannot be simply explained by sperm analysis [Männikkö et al. 2005; Miyara et al. 2003] including oocyte factors [Kilic et al. 2008;

Table 3. Oocyte fertilization outcome, embryo quality, and pregnancy rate.

	Microdrop insemination group	Open insemination group	*p* Value
Cases (*n*)	573	602	–
Fertilization failure cases* *n* (%)	68/573 (11.9%)	20/602 (3.3%)	<0.001
Total fertilization failure *n* (%)	28/573 (4.9%)	7/602 (1.2%)	<0.001
Low fertilization *n* (%)	40/573 (7.0%)	13/602 (2.2%)	<0.001
Normal fertilization rate per oocytes ($\bar{x} \pm$ SD, 2 PN%)	59.3 ± 21.8	62.0 ± 21.3	0.374
Polyspermy rate per oocytes ($\pm$ SD, %)	6.5 ± 8.4	10.1 ± 40.1	0.283
Normal fertilization rate per mature oocyte ($\pm$ SD, %)	66.1 ± 13.7	67.3 ± 11.2	0.423
Fertilization failure rate per mature oocyte ($\pm$ SD, %)	21.7 ± 8.3	20.3 ± 7.9	0.919
Polyspermy rate per mature oocyte ($\pm$ SD, %)	5.6 ± 7.7	5.8 ± 11.9	0.864
Cleavage rate ($\bar{x} \pm$ SD, %)	96.1 ± 11.1	96.1 ± 8.1	0.990
Good embryo rate ($\bar{x} \pm$ SD, %)	73.9 ± 35.7	76.1 ± 23.2	0.611
Transferred embryos ($\bar{x} \pm$ SD)	2.21 ± 0.55	2.26 ± 0.61	0.333
Clinical pregnancies *n* (% per embryo transfer)	223/532 (41.9%)	256/592 (43.2%)	0.654

*The fertilization failure cases included total fertilization failure cases (TFF, 0% oocytes fertilized) and low fertilization cases (<25% oocytes fertilized).

Zhang et al. 2013]. However, in the present study a relatively large number of non-male factor patients were included and the results demonstrated that the fertilization failure rate was different between the insemination methods (microdrop vs. open), although the normal fertilization rate (2 pronuclei (PN)%) and abnormal fertilization rate (≥3PN%) were not significantly different.

The primary differences between the microdrop and open insemination methods are the volume of medium (100 µl vs. 1 ml) and the presence of an oil overlay which is the major difference. Though it had passed the mouse zygote bioassay and had been further washed and equilibrated, there still remains the possibility that trace amounts of unknown material were released into the insemination medium, which might interfere with fertilization [Hughes et al. 2010; Sifer et al. 2009]. For example, Otsuki et al. [2007] indicated that peroxidation of mineral oil used in the droplet culture was detrimental to fertilization and embryo development. Therefore, the increased fertilization failure rate in the present study might be due to this or another effect. For example, the microdrop has a relative higher surface area to volume ratio, which may result in an increased loss of lipid soluble components, specifically steroid hormones, present in the medium into the oil [Shimada et al. 2002]. This may include progesterone from the cumulus matrix that had been shown to enhance some sperm function including motility and hyperactivation [Sagare-Patil et al. 2013]. The transfer of these and other components into the oil may have a detrimental effect on sperm function.

One problem regarding oocytes surrounded by hundreds of cumulus cells and thousands of sperms during small volume microdrop insemination was the potential for the generation of an acidic environment [Morgan et al. 1995; Wiemer et al. 1993]. In this environment, oocytes, cumulus cells, and sperm consume oxygen together. Swain [2010] showed the influence of pH and buffer capacity on gamete and embryo quality and that a pH optimum was required for superior clinical outcomes [Swain 2012]. At present, it was not clear whether the reduced volumes of insemination medium and decreased pH value were limiting factors for oocyte fertilization and later embryo development. For example, some studies have shown media pH can impact sperm motility [Hamamah and Gatti 1998]. But Harraway et al. [2000] have shown that the pH of semen does not play an important role in the outcome of intrauterine insemination.

One must also consider that the different manipulation procedures associated with each insemination technique itself might also contribute in part to the different fertilization outcome between the 2 groups, as oocytes might be physically activated by excessive manipulation [Marchesi et al. 2012; Xie et al. 2007]. In the present study, oocytes were transferred to the sperm suspension microdroplets for insemination in the microdrop group, but oocytes in the open insemination group had not been manipulated (sperm suspension was added directly to the wells incubated with oocytes).

IVF-ET outcome

Although the fertilization failure rate was different between the two groups, the normal fertilization (2 PN) rate and the normal fertilization rate of mature oocytes were both not significantly different between the two groups (Table 3). The polyspermy rate was also not significantly different between the two groups (Table 3). The embryo cleavage rate, the good quality embryo rate did not differ significantly between the two groups (Table 3). The clinical pregnancy rate per transfer was 41.9% in the microdrop insemination group and 43.2% in the open insemination group ($p = 0.654$) (Table 3). This indicated that the two methods themselves may not influence the final outcome of IVF-ET.

Microdrop insemination has several limitations but it surely has several potential advantages. Small volumes of insemination media require a lower total number of sperm to fertilize oocytes (as insemination is based on concentration), which is especially beneficial for patients presenting with oligospermia and normal motility [Suh et al. 2006]. Perhaps the small microdrop volume concentrates autocrine and paracrine factors secreted by embryos, oocytes, and cumulus cells stimulating embryo development [Kawamura et al. 2007, 2012]. An oil cover can stabilize the culture environment and protect it from contamination. Moreover, the possible detrimental effects of the microdrop insemination method on fertilization outcome were apparently not detrimental to the

ensuing embryo development because pregnancy rates and embryo quality were not significantly different between the two groups.

The physiology of fertilization is not well described [Pregl Breznik et al. 2013].The processes, such as zona pellucida binding and oocyte activation, are yet uncertain. It was not determined whether the increased fertilization failure rate was related to the specific brands or lots of mineral oil, or with the technique of microdrop insemination itself. The effects of volume (insemination medium) and mineral oil on the fertilization outcome along with experimental design need to be considered. For example, in addition to sperm factors, there were several unknown causes of fertilization failure, such as oocyte derived causes [Swain and Pool 2008]. If each patient could have her oocytes split between the two methods to control for differences in age, stimulations, oocyte derived causes, etc, a more concrete conclusion could be drawn.

In conclusion, our study showed that the microdrop insemination method had a higher rate of fertilization failure compared with the open insemination method. However, as a highly complicated process involving many extrinsic and intrinsic factors, the effects of the two insemination methods on the rate of fertilization failure needs to be assessed further.

Materials and Methods

Ethics

All issues concerning the experimental setups and evaluation techniques have been approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University.

Patient selection and assignment

This prospective study was based on a cohort of consecutive infertile couples undergoing conventional IVF treatments at the Reproductive Medicine Center, the First Affiliated Hospital of Sun Yat-sen University, during the period from January 2012 to June 2013. Couples in which the male partner had normal sperm concentration and motility according to the World Health Organization criteria [Murray et al. 2012] and normal morphology and functional test (acrosome reaction test) were asked to participate in the study. Patients with <4 oocytes retrieved and with the diagnosis of PCOS or PCO were excluded from the study. All the patients did not use hormone supplements within three months before the IVF-ET protocols and did not have any medical conditions affecting the quality of the oocytes, such as autoimmune diseases.

Patients were randomized into two groups according to the day of oocyte retrieval and insemination. If oocytes were retrieved on odd numbered days, they were inseminated with the microdrop method with oil overlay (group 1/microdrop insemination group, $n = 573$). If oocytes were retrieved on even numbered days, they were inseminated with an open insemination method without oil overlay (group 2/ open insemination group, $n = 602$). Each patient contributed just one cycle. All the couples signed written informed consent.

Protocol of ovarian stimulation and oocyte retrieval

Downregulation using a gonadotropin-releasing hormone analogue (GnRHa, Decapeptyl PL 3, Ipsen, Paris, France) was performed in the luteal phase of each patient, at a dose of 1.0 mg. When a blood test indicated pituitary suppression, recombinant follicle stimulating hormone (rFSH, Gonal F, Merck Serono, Switzerland) was administered until at least two follicles reached a diameter of $\geq$18 mm. Patients then received 250 μg of rHCG (Ovidrel, Merck Serono) intramuscularly 36 h before the scheduled follicular aspiration.

After aspiration, oocytes were washed in culture medium and placed in groups in 1 ml of equilibrated HTF-fertilization medium (Sage BioPharma, Bedminster, NJ, USA) in a 1-well dish (3037, Falcon) and incubated for 3–5 h at 37 °C, 6% CO_2 atmosphere. If more than 15 oocytes were retrieved, another dish was used.

Sperm preparation

Semen samples were collected by masturbation. After liquefaction at room temperature for 30–60 min, semen samples were assessed for sperm density, motility, and morphology. A 2-layer Pureception gradient (80% and 40%) (Sage BioPharma, Bedminster, NJ, USA) centrifugation followed by a swim-up procedure was used for sperm selection.

Insemination and fertilization

Group 1: Microdrop insemination with oil cover

At the end of oocyte retrieval, sperm were diluted to 0.3×10^6/ml with HTF-fertilization. Then 100 μl droplets of the sperm suspension was placed into a 60 mm petri dish (3002, Falcon) covered with liquid oil and equilibrated in the incubators. One-two oocytes were transferred into each microdroplet containing motile sperm after 3–4 h pre-incubation in HTF medium *in vitro.* Oocytes and sperms were incubated 16–20 h until the fertilization evaluation [Gianaroli et al. 1996].

Group 2: Open insemination without oil overlay

After a 3–5 h *in vitro* pre-incubation of oocytes, a measured volume of the sperm suspension was added into each single well to get a final concentration of 0.3×10^6 progressively motile spermatozoa per ml/well. No more than 15 oocytes were transferred into each dish containing motile sperms after the 3–4 h pre-incubation in HTF medium *in vitro*. Oocytes and sperm were incubated 16–20 h until evaluating fertilization.

Assessing fertilization, embryo morphology, and embryo transfer

Assessment of fertilization, the culture procedure, embryo morphology, cryopreservation, and embryo transfer procedure were the same between the 2 groups as a function of the method of insemination.

Observation of 2PN and 2 polar bodies (2PB) were performed in both groups 16–20 h after the exposure of oocytes to spermatozoa. Normal fertilization was characterized by 2 visible, distinct PN and 2PB. Polyspermy was

defined as more than 2 visible PN. No visible PN were defined as unfertilized oocytes. Because the oocyte-cumulus complexes were inseminated using a conventional IVF method, mature oocytes were finally confirmed when 2PB were seen on the first day after the oocytes were retrieved. Cleavage was assessed on the third day after oocyte recovery, and embryo quality was evaluated using the modified criteria of Cummins and Breen [Cummins et al. 1986]. A good quality embryo was defined as, on day 3, after oocytes retrieved, the embryos contain 6–9 cells or were in compacted stage with less than 20% fragment. According to the guideline of the Ministry of Health of China, no more than three embryos were selected for transferring into the uterine cavity. Supernumerary good quality embryos were cryopreserved. Luteal support was achieved using progesterone in oil, 40 mg/day i.m.

Statistical evaluation

The Variance Analysis and $\chi 2$ test were performed for statistical analysis by using the SPSS 13.0. A p value less than 0.05 was considered statistically significant.

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Declaration of interest

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Author contributions

Participated in the study design and execution: Y-BL; Participated in the study design and had overall supervision: TL; Participated in the study design and the drafting of the manuscript: Q-YM; Participated in the study execution and interpretation of data: LL, JO. All authors read and approved the final manuscript.

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