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ASSISTED REPRODUCTION TECHNOLOGIES

Correlation between follicular diameters and flushing versus no flushing on oocyte maturity, fertilization rate and embryo quality

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Abstract
Objective To determine (a) the correlation between follicular sizes, oocyte maturity, normal fertilization rate, cleavage and embryo quality; and (b) to establish whether oocytes recovered with or without follicular flushing have different developmental competence.
Design Prospective observational study.
Setting Academic medical center.
Patients Forty nine cycles (37 ICSI and 12 IVF).
Interventions Measurement of 360 follicular diameters on the day of egg retrieval and classification into three groups Group A (mean diameter 12–14.5 mm.), group B (mean diameter 15–18 mm.) and group C (diameter >18.5 mm.).
Main outcome measure Correlation between follicular size at the time of retrieval and oocyte maturity, fertilization and cleavage rate in 226 oocytes (163 ICSI and 63 IVF). Developmental competence of oocytes retrieved with flushing versus non flushing.
Results Almost all (99 %) of the oocytes recovered from follicles of group C were in metaphase II as opposed to 80 % in group A and 81 % in group B ($p<0.01$). Overall there was a progressive and significant increase in fertilization rates from group A follicles to group C (47 % vs. 67 %, p 0.05). Overall 53 % of oocytes retrieved from group A follicles showed either no fertilization or abnormal fertilization versus 27 % in group C (p 0.05). The oocyte recovery rate with follicular flushing improved from group A to group B and to group C follicles (65 % vs. 49 % vs.37 % respectively $p<0.01$). There were no differences in rates of immature oocyte, fertilization, abnormal or not fertilization and cleavage.
Conclusions The results of this study shows that: a) Follicles larger than 18 mm at retrieval have consistently mature oocytes with a higher rate of fertilization; b) Small size follicles are still capable of containing mature oocytes, but their rate of abnormal or no fertilization is high; c) Oocytes recovered with flushing are still able to produce embryos with full developmental competence.

Keywords Follicle size · Flushing · In vitro fertilization · Ovarian stimulation · ICSI

Capsule Oocyte maturity according to follicle size and flushing.

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Introduction

In preparation for cycles of assisted reproduction (ART), controlled ovarian hyperstimulation is used for follicular recruitment. However, not all follicles develop in synchrony, and it is a common occurrence that within a specific cohort there are follicles of different diameters. Thus, at the time of retrieval, oocytes are collected from follicles of varying sizes, and this variation appears to coincide with a correlation between follicular size and stage of oocyte development and maturity. A positive correlation between follicular size and the presence of metaphase II oocytes has been reported [1, 2]. In addition, previous literature has suggested that oocytes from follicles with a mean diameter greater than 12 mm have significantly higher fertilization and cleavage rates [3, 4]. Dubey et al. [5] suggested that measurement of follicular size before retrieval might be the best indicator of the fertilization potential of oocytes in conventional IVF cycles. However

Table 1 Correlation between follicular group size and overall oocyte recovery

	Group A (<15 mm.)	Group B (15–18 mm.)	Group C (>18 mm.)
No. of Follicles	110	103	147
No. of Oocytes Recovered	60	62	104
% Oocyte Recovery Rate	54.5 %*	60.2 %	70.7 %*

*Chi Square=6.5, df=2: $p=0.01$

there are scant data correlating follicle size with oocyte maturity in ICSI cycles [6].

It is commonly known that a proportion of oocytes collected remain meiotically immature at the germinal vesicle (GV) or metaphase I (MI) stage. This proportion fluctuates from around 15 to 20 % [7, 8]. Some MI oocytes can undergo the final stages of meiotic maturation spontaneously and may progress to the metaphase II (MII) stage within a few hours of in vitro culture and thus can also be utilized for the ICSI procedure.

However these in vitro matured oocytes yield lower fertilization rates [9, 10], abnormal embryonic development [11] and lower implantation rates [9] than in vivo matured oocytes. Therefore, it becomes important for ICSI cases to retrieve oocytes that are mostly in metaphase II and to clarify what is the follicular diameter likely associated with mature oocytes. In addition to follicular size and oocyte maturity, another point of controversy in the literature is whether oocytes collected after follicular flushing have less likelihood of normal fertilization than those obtained without flushing, and whether the resulting embryos would have different developmental potential.

The aim of this study was thus two-fold: first to correlate follicular sizes with oocyte maturity, fertilization and cleavage, and embryo quality rate; and second to analyze whether oocytes retrieved with follicular flushing resulted in a lower maturity, fertilization and embryo quality rate.

Materials and methods

Data were prospectively obtained from 49 consecutive normogonadotropic patients undergoing ICSI ($n=37$) and conventional IVF (controls $n=12$) cycles at the Yale Fertility Center. Ovarian stimulation was accomplished exclusively with r-FSH after luteal phase leuprolide acetate (Lupron, Tap Pharmaceuticals, and IL).

The follicular phase was monitored by transvaginal ultrasound and serum estradiol measurements. Human chorionic gonadotropin (HCG 10.000 IU) was given when at least two follicles had mean diameters of 20 mm. Oocyte retrieval was scheduled 36 h after HCG administration and each patient had five follicles of different diameters identified as included in the study.

On the day of the oocyte retrieval, before follicular aspiration was started, the size of each follicle allocated for the study was measured by transvaginal ultrasound as a mean of two diameters. Follicles were classified into three groups according to their diameter: group A (mean diameter <15 mm), group B (mean diameter 15–18 mm) and group C (mean diameter >18 mm). Of note, since follicle size was also available from the measurements obtained on the day of HCG administration, it was noted that each follicle grew about 2 mm in size during the 36 h. In other words a follicle that measured 14 mm at HCG day it was 16 mm on the day of retrieval. The follicular aspiration was carried out by using a double lumen 17 G Cook needle (Cook; OB/GYN Spencer, IN) with suction pressure set at 100 mmHg. If an oocyte was not recovered, the follicle was flushed twice with 2 ml of HEPES-buffered HTF media (Irvine Scientific, CA).

Oocytes were independently recorded according to the follicular size and whether obtained by flushing or not. The same provider (author SM) performed all the follicular measurements and the same provider (author PP) performed all the oocyte retrievals. In the embryology laboratory, all the oocytes were kept individually and classified according to their nuclear maturation as germinal vesicle (GV), metaphase I (MI), or metaphase II (MII) for the ICSI patients. For the

Table 2 Correlation between follicular group size and outcomes in both ICSI and IVF groups

Group	Total number of oocytes	GV	MI	MII	Fertilization rate	Abnormal fertilization	Not fertilized	Cleavage rate
A (<15 mm)	60	5	7	48 (80.0 %)	47 %**	15 %	38 %	81 %
B (15–18 mm)	62	4	8	50 (80.6 %)	52 %	12 %	36 %	67 %
C (>18 mm)	104	0	1	103 (99.0 %)*	67 %**	9.7 %	23 %	79 %

*Chi Square=20.0, df=2: $p<0.001$ **Chi Square=4.2: $p=0.04$

Table 3 Correlation between follicular group size and outcomes in IVF patients (Oocytes=63)

Group	Total number of oocytes	GV	MI	MII	Fertilization rate	Abnormal fertilization	Not fertilized	Cleavage rate
A (<15 mm)	13	3	1	9 (69.2 %)	40 % (4/10)	20 % (2/10)	40 % (4/10)	100 % (4/4)
B (15–18 mm)	13	0	4	9 (69.2 %)	46 % (6/13)	0 %	54 % (7/13)	67 % (4/6)
C (>18 mm)	37	0	0	37 (100.0 %)*	73 %** (27/37)	11 % (4/37)	16 % (6/37)	89 % (24/37)

*Chi Square=13.0, df=2: $p<0.01$ **Chi Square=8.8, df=2: $p=0.01$

conventional IVF patients (controls) oocytes were classified the following day. The embryologists were not aware of the follicle size of the corresponding oocytes.

Following standard ICSI or IVF procedure [12, 13], oocytes were individually cultured in IVF media (IVF-30; Vitrolife, Englewood, Colorado, USA) for 16 to 20 h after which they were examined for normal fertilization. Normal fertilization was determined by the presence of two pronuclei (2 PN) and two polar bodies. The number of oocytes showing one pronucleus (1PN) or abnormal fertilization, i.e. appearance of three pronuclei (3PN), was also recorded.

The quality of embryos was evaluated daily up to transfer day by morphologic criteria (fragmentation, granularity, number and size of blastomeres) and graded on a scale of 1–5 [14, 15]. Group 1, or excellent quality, embryos were defined as embryos in which all blastomeres were of an equal size without any nuclear fragments. Group 2, or good quality, embryos had blastomeres of equal size and a maximum of 20 % nuclear fragments. In the 3rd category, no fragmentation was present but blastomeres were uneven. In group 4 embryos, blastomeres could still be visible but fragments present in 50–60 % of the volume of embryo and in group 5 embryos, blastomeres could not be seen and there was 70–100 % of nuclear fragmentation.

Statistical analysis

For comparison among groups, results were analyzed with Chi-square tests with either 1 or 2° of freedom (df) when appropriate. When the P value was <0.05, the difference was considered significant.

Results

Three hundred and sixty follicles from 49 cycles (37 ICSI and 12 IVF) were measured.

There was a significantly higher recovery rate for oocytes from large follicles (Group C) compared to small follicles (Group A, $P<0.01$) (Table 1).

A total of 226 oocytes were prospectively evaluated for oocyte maturity (Table 2) and 99 % of oocytes recovered from follicles of group C were in metaphase II as opposed to 80 % in group A and 81 % in group B ($P<0.01$). In group A ($n=12$), 42 % of the immature oocytes were GV's and the remaining MI. In group B ($n=12$) 33 % were GV's and 67 %MI. The fertilization rate of mature oocytes of group A was 47 % versus 67 % for mature oocytes of group C ($P=0.01$). The rate of oocytes abnormally fertilized (i.e. 1PN and 3PN) or not fertilized was higher in group A than B or C (53 % vs.48 % and 33 %, respectively (the difference between oocytes of group A vs. oocytes of group C was statistically significant, $P=0.01$).

The fertilization rate in relation to follicular size and insemination method (IVF or ICSI), indicated that there was a progressive and significant increase in fertilization rates from group A follicles to group C in IVF cycles (40 % vs. 73 %, $P<0.05$) (Table 3). In ICSI cycles the fertilization rate also increased from group A to C, but it did not reach statistical significance (49 % vs. 64 %, $P=0.2$), as shown in Table 4.

In IVF cycles a significantly higher proportion (60 %) of the oocytes from group A showed either no or abnormal fertilization when compared to oocytes of group C (27 %), ($P<0.05$). In ICSI cycles, 51 % of the oocytes from group A

Table 4 Correlation between follicular group size and outcomes in ICSI patients (Oocytes=163)

Group	Total number of oocytes	GV	MI	MII	Fertilization rate	Abnormal fertilization	Not fertilized	Cleavage rate
A (<15 mm.)	47	2	6	39 (83.0 %)	49 % (22/45)	13 % (6/45)	38 % (17/45)	77 % (17/22)
B (15–18 mm.)	49	4	4	41 (83.7 %)	53 % (24/45)	16 % (7/45)	31 % (14/45)	67 % (16/24)
C (>18.5 mm.)	67	0	1	66 (98.5 %)*	64 %** (43/67)	9 % (6/67)	27 % (18/67)	72 % (31/43)

*Chi Square=9.8, df=2: $p=0.01$ **Chi Square=2.9, df=2: $p=0.2$

Table 5 Correlation between follicular group size and overall embryo development grades

Group	Total number	Grade I	Grade II	Grade III	Grade IV	Grade V
A (<15 mm.)	26	19 %	65 %	8 %	8 %	0 %
B (15–18 mm.)	30	30 %	37 %	13 %	17 %	3 %
C (>18 mm.)	70	24 %	60 %	10 %	4 %	1 %

Embryo grades showed no significant differences

and 36 % of the oocytes from group C showed either no or abnormal fertilization (P=NS).

There was no significant difference between cleavage rates on day 3 between embryos originating from oocytes of patients in group A versus group C and whether obtained by IVF or ICSI.

Altogether, the 126 embryos that were assessed for quality on day 3, showed no significant difference between the follicular diameter and the rate of embryos scored as good (Table 5).

A total of 162 oocytes in the ICSI group were evaluated according to whether obtained by follicular flushing (n=68) or not (n=94). The oocyte recovery rate with follicular flushing significantly improved from group A to group B and to group C follicles (65 % vs. 49 % vs. 37 % respectively, p<0.01).

There were no significant differences in immature oocyte rates between flushed versus not flushed groups of follicles (6 % vs.5 %, respectively, P=NS), fertilization rates (64 % vs. 60 % respectively, p=NS), abnormal or not fertilized rates (35 % vs. 40 % respectively, P=NS) and cleavage rates (72 % vs. 77 %, respectively, P=NS) (Table 6).

Discussion

In the present study, we found a significantly higher oocyte recovery rate from large compared to small follicles. In addition, the aspiration of follicles greater than 18 mm. provided the highest probability of retrieving mature oocytes (MII), confirming previous publications [2, 3, 6].

The recovery of MII oocytes and their fertilization rates were different according to follicle size regardless of the method of insemination. Although follicles of smaller size (group A) are still capable of containing mature oocytes, their fertilization rate is lower than similar MII oocytes obtained from larger size follicles. In addition, the rate of abnormal fertilization or no fertilization is also higher for oocytes retrieved from smaller follicles. The clinical consequence of this observation is to trigger ovulation in patients who are candidates for ICSI when a larger number of follicles are beyond 15 mm of diameter so as to maximize the number of mature oocytes and normal fertilization. Interestingly, a proposed use of polarized microscopes has been able to identify MII oocytes that have a non-developed spindle [16]. It could be postulated that the MII oocytes that originate from smaller follicles may be those that take longer to mature in spindle formation or have arrested spindle dynamics.

Another aim of our study was to assess whether oocytes obtained by follicular flushing were less capable of fertilization and cleavage as opposed to oocytes retrieved without flushing.

Over half of assisted reproductive clinics use follicular flushing to obtain oocytes in addition to direct aspiration of fluid during oocyte retrieval [17]. The optimum number of follicular flushing appears to be four times as reported by Bagtharia et al. [18]. They found that about 40 % of the oocytes are retrieved with primary aspiration without follicular flushing, while up to 82 % of oocytes are retrieved with two flushes and 97 % of oocytes up to four flushes and only 3 % of the remaining oocytes are retrieved with 5th and 6th flush [18]. In contrast a recent Cochrane review showed that with the utilization of proper retrieval techniques, most of the oocytes are retrieved without the need for follicle flushing [19, 20]. However the issue of whether flushing should be not performed is still very much controversial and hotly debated (see discussions posted on www.IVFworldwide.com accessed January and February 2013).

To evaluate the importance of follicular flushing, some studies have also compared the reproductive potential of oocytes obtained from follicular fluid to those obtained from follicular flushing. They demonstrated similar fertilization and implantation rates suggesting that the practice of follicular flushing improves the pregnancy rate [21]. Furthermore,

Table 6 Correlation between flushed and non-flushed follicular group sizes and outcomes (ICSI)

Group	Total number of oocytes	GV	MI	MII	Fertilization rate	Abnormal or no fertilization	Cleavage rate
Flushed	68	0	4	64 (94 %)	64 %	35 %	72 %
Non Flushed	94	4	5	85 (90 %)	60 %	40 %	77 %

Flushing showed no significant advantage

Knight et al. found no difference in the outcome of ART from oocytes collected with aspiration of follicles accomplished by flushing [22].

In this study we have shown that oocytes obtained by follicular flushing (when no oocyte was obtained in the initial aspirate) do not show impaired fertilization or abnormal cleavage rates, similar to the findings of other investigators [21, 22]. Flushing of follicles increased the total number of collected oocytes and this may be important particularly for patients with small number of follicles or for patients with a cohort of small size follicles.

In conclusion, our results indicate that there is a significantly higher recovery rate for oocytes from follicles larger than 18 mm measured at the time of retrieval. These follicles were 16 mm in diameter at the time of hCG. Also follicles larger than 18 mm have consistently more mature oocytes with a higher rate of fertilization. However, even oocytes from follicles <15 mm. achieved normal fertilization. This may allow the total number of good quality and transferable embryos to be increased. Follicular flushing increases oocyte yield per follicle and flushing of small follicles increases total number of oocytes similar to previous studies. We also found that follicular flushing improves the oocyte retrieval rate without an apparent decrease in oocyte maturity or fertilization rate and furthermore embryos derived from those oocytes still retain full developmental competence.

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