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ASSISTED REPRODUCTION TECHNOLOGIES

Effect of follicular flushing on reproductive outcomes in patients with poor ovarian response undergoing assisted reproductive technology

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Abstract
Purpose The purpose of this study is to investigate the impact of follicular flushing on the number of oocytes retrieved, oocyte maturity, fertilization rate, embryo development, and pregnancy rate of poor ovarian responders (POR).
Methods Retrospective study of 524 cycles of 384 patients with POR submitted to assisted reproductive technology (ART) and who had follicular flushing during oocyte retrieval was used in the study. We included patients with <5 oocytes at oocyte retrieval (POR group) and matching the Bologna criteria.
Results POR patients had a mean age of 38.2 ± 4.2 years. A total of 1355 follicles (mean = 3.5 ± 1.6) were aspirated and 1040 oocytes recovered, with 709 (68.2%) obtained by direct aspiration and 331 (31.8%) by follicular flushing. We found a difference between the total number of oocytes and the number of aspirated oocytes. Overall pregnancy rate was 22%. Association was observed between pregnancy rate and the number of oocytes retrieved, the number of MII oocytes, and the number of embryos transferred. The patients matching the Bologna criteria had a mean age of 38.9 ± 3.9 years. A total of 309 follicles were aspirated (mean = 3.1 ± 1.5) and 242 oocytes recovered, with 156 (64.5%) obtained by direct aspiration and 86 (35.5%) by follicular flushing. There was a significant difference between the total number of oocytes and the number of aspirated oocytes. Overall pregnancy rate was 12.1%. There was no association between the pregnancy rate and the number of oocytes retrieved, the number of MII, and the number of embryos.
Conclusions Follicular flushing might be a suitable alternative to increase the number of oocytes and pregnancy rates in patients with POR.

Keywords Poor ovarian response · Follicular flushing · Oocyte retrieval · Ovarian stimulation · Assisted reproduction techniques

Introduction

Poor ovarian response (POR) occurs in approximately 9% of women undergoing in vitro fertilization (IVF) [1] Although different controlled ovarian hyperstimulation (COH) protocols have been proposed for POR, there are still women who respond poorly to gonadotropins, thus resulting in few oocytes at retrieval, reduced number of embryos for transfer, and unsatisfactory pregnancy rates; and no consensus has been reached for the best alternative to increase the number of oocytes and embryos available for transfer in poor responders [2, 3].

An alternative to increase the number of oocytes is follicular flushing during oocyte retrieval (OR), a technique easily performed using double-lumen needles. Follicular flushing improves the odds to overcome oocyte retention within the follicle during direct aspiration or in the collection system, thereby increasing the number of recovered oocytes [4, 5].

Several studies have described the use of double-lumen needle during oocyte retrieval in normal responders. Early studies using this technique showed an increase in the number of recovered oocytes after follicular flushing in 20% of the cases, when compared with aspiration [4, 5]. Others, however, did not observe differences in the number of retrieved oocytes, fertilization rates, embryo quality, or pregnancy rates [6, 7].

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However, only a few studies have investigated POR patients and analysed the effectiveness of this oocyte retrieval strategy on reproductive outcomes. Levens et al. (2009) studied POR but they excluded women with <4 follicles. Mok-Lin et al. (2013) also included very poor responders (1 to 4 follicles) and suggested that follicular flushing might be associated to lower implantation and pregnancy rates. Therefore, due to the scarceness of data regarding follicular flushing efficacy in POR patients, we proceeded to investigate the impact of follicular flushing on ART outcomes in these patients.

Materials and methods

We performed a retrospective study evaluating 524 in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles of 384 patients diagnosed with POR who had been subjected to follicular flushing during oocyte retrieval for assisted reproductive technology (ART) treatment, from January 2002 to December 2012. The study was approved by the National Research Ethics Committee (CONEP - No 617.430). An informed consent was not required. Only patients who had at least one embryo transferred were included. Patients with severe male factor infertility were not included. Two parallel analyzes were conducted. The first included all patients with a diagnosis of POR using the criteria of <5 oocytes at oocyte retrieval (POR group), and the second included patients diagnosed with POR according to the Bologna criteria (Bologna criteria group) [8]. We evaluated the impact of follicular flushing on the number of oocytes retrieved, oocyte maturity, fertilization rate, embryo development, and clinical pregnancy rate.

Ovulation induction

All patients were subjected to the same protocols for ovulation induction, using the same hormones and the same criteria for dose tailoring. Ovulation induction was performed using the antagonist or the long protocols. The antagonist protocol initiated with daily administration of the recombinant follicle-stimulating hormone (rFSH, Gonal-F, Merck-Serono, Brazil) on day 2 of the menstrual cycle. The dose of rFSH was tailored according to ovarian response measured by estradiol serum concentrations, and follicular growth was monitored by vaginal ultrasound. When follicles reached 14 mm, patients started receiving gonadotropin releasing hormone (GnRH) antagonist (Cetrotide, MerckSerono, Brazil) associated with rFSH. For the long protocol, treatment started with a subcutaneous administration of 3.75 mg of GnRH agonist (Gonapeptyl, Ferring, Brazil) on day 21 of their menstrual cycle, to suppress the pituitary function. To confirm downregulation, serum estradiol concentrations and vaginal ultrasound were performed approximately 10 days later. If the estradiol concentration was <30 pg/ml and the ultrasound showed an endometrial thickness of <3 mm, patients were considered ready to start ovulation induction. After confirmation of suppression, patients underwent ovulation induction with daily administration of rFSH. In both protocols, oocyte maturation was induced with recombinant human chorionic gonadotrophin (hCG, Ovidrel, Merck-Serono, Brazil) when at least two follicles reached a mean size of 17 mm with concordant estradiol levels (approximately 200 pg/ml).

Oocyte retrieval

Oocyte retrieval was performed approximately 34 h after rhCG injection by vaginal ultrasound-guided aspiration. The same person performed all retrievals. We used a double-lumen 17 gauges follicular puncture needle (Casmed, UK) connected to a vacuum pump (Rocket Craft suction pump, Rocket Medical, UK) with pressure of approximately 100 mmHg. The aspirated follicular fluid was placed in 14-ml test tubes (Falcon, USA). If an oocyte was not identified in the follicular fluid, 3 ml of HEPES buffered culture medium (Sigma, USA) was injected into the follicle, and the intrafollicular flushing was reaspirated in a different test tube. The procedure was repeated until the oocyte was identified or up to a maximum of ten times.

ICSI and embryo culture

Collected oocytes were placed in petri dishes (Falcon–BD, USA) containing 20 ml of Earle's balanced Salt Solution (EBSS) culture medium (Sigma, USA) supplemented with 10% synthetic serum substitute (SSS) (Irvine Scientific, USA) and 0.47 mM pyruvate (Sigma, USA) and covered with mineral oil (Sigma, USA). Oocytes were kept for 2 h in the incubator atmosphere of 6% carbon dioxide (CO2) at 37 °C, and denuded with 80 IU/ml hyaluronidase (Sigma, USA) and mechanical pipetting. Oocytes' maturity was assessed by the presence or absence of a germinal vesicle (GV) or the first polar body (PB) using a stereomicroscope (×400) (Nikon, Japan). Metaphase II (MII) oocytes were inseminated by ICSI as reported previously [9]. Briefly, after complete removal of the corona cells, oocytes were placed in a fresh droplet of culture medium. Micromanipulation procedure was carried out on a heated stage of an inverted microscope (Nikon Diaphot, Japan) adapted with a pair of hydraulic micromanipulators and a motor-driven course control (Narishige, Japan) at ×400 magnification. A single sperm was aspirated into the injection micropipette from a drop of HEPES-buffered medium (Sigma, USA) containing 10% polyvinylpyrrolidone (PVP, Irvine, USA). The holding micropipette was lowered and the oocyte held in place. The injection pipette was then pushed through the zona pellucida into the cytoplasm and a single spermatozoon was injected. Next, the inseminated oocytes were transferred to droplets of culture medium under

mineral oil in petri dishes at 37 °C and 6% CO_2. On the following day, i.e. 17–19 h later, the oocytes were checked for normal fertilization. The embryos were cultured in EBSS with 10% SSS under mineral oil in a petri dish (Falcon–BD, USA) at 37 °C and 6% CO_2. They were checked daily for standard morphological analysis until transfer [10].

Embryo transfer and luteal phase support

Embryo transfers were performed on days 2, 3, or 5 after ICSI using a soft transfer catheter (Sydney IVF, Cook, Australia) under abdominal ultrasound guidance. Luteal phase was supported with vaginal progesterone (Crinone, Merck-Serono, Brazil) starting on the evening of day 1 after oocyte retrieval [11]. Embryos not selected for transfer were cryopreserved on the same day.

Outcome

Pregnancy was determined by serum β-hCG levels measured 14 days after oocyte retrieval. Clinical pregnancy was defined by the observation of intrauterine embryo heart-beat by 7 weeks of gestation. The clinical pregnancy rate was calculated as the ratio of the number of clinical pregnancies to the number of embryo transfers. The main outcome measure was clinical pregnancy rate. Number of retrieved oocytes, oocyte maturity, fertilization rate, and embryo development were the secondary outcome measures.

Statistical analysis

The Student's *t* test was used to compare two independent groups as a scalar variable. The Levene test was used to verify the homogeneity of variances of each variable for each group. Due to the heterogeneity of variances, we used the Student's *t* test values observing non-equal variances. The association/relationship/dependence between two categorical variables of interest was determined using the chi-square test. In the event of a significant association between two variables of interest, we assessed the statistical odds ratio (OR). Fisher's exact test was adopted to compare the groups as the proportion of occurrence of a specific event. The chi-square test Mantel-Haenszel was applied to evaluate association/linear relationship between two categorical variables. The OR and 95% confidence interval (CI) of each of the factors were calculated. Statistical significance was established when $p < 0.05$.

Results

A total of 384 patients performing 524 cycles of ART/ICSI were included in the study. Records from 313 patients (386 ART cycles) were analysed, as 71 patients were excluded (138 cycles) as they did not met inclusion criteria or had incomplete records. Clinical characteristics of patients are summarized in Table 1. The mean age of the patients was 38.2 ± 4.2 years (22–46) in the POR group and 38.9 ± 3.9 years (28–46) in the Bologna criteria group. The mean time of infertility was similar in both groups: 5.2 ± 5.1 and 5.1 ± 4.5 years for the POR group and the Bologna Criteria group, respectively. Advanced patient age was the main cause of infertility in both groups (57.5 and 63% for POR and Bologna Criteria groups, respectively). Regarding the POR group, the antagonist protocol was used in 74.8% of the cycles ($n = 289$), while 25.2% of cycles ($n = 97$) received the long protocol. Concerning the Bologna criteria group, the antagonist protocol was used in 87% ($n = 87$) of patients while 13% ($n = 13$) received the long protocol.

In the POR group, 1355 follicles (mean = 3.5 ± 1.6) were aspirated (size range—16–18 mm), and 1040 oocytes were recovered, with 709 (68.2%) obtained by direct aspiration and 331 (31.8%) by follicular flushing. We found a significant difference between the total number of oocytes (aspirated and flushed) and the number of oocytes that were only aspirated ($p < 0.001$) (Table 2). A total of 812 oocytes (78.1%) were classified as MII, 225 (21.6%) as Metaphase I (MI), and three (0.3%) as GV. Normal fertilization was confirmed in 74.7% (775) of the inseminated oocytes and development to cleavage stage embryos occurred in 74.9% (581), with 94.6% being considered of good quality.

Table 1 Clinical characteristics of poor ovarian responder patients in assisted reproduction cycles and submitted to follicular flushing for oocyte retrieval

	POR $n = 386$	Bologna Criteria $n = 100$
Age (years)	38.2 ± 4.2	38.9 ± 3.9
Primary infertility	85.2%	89%
Secondary infertility	14.8%	11%
Duration of infertility (years)	5.2 ± 5.1	5.1 ± 3.4
Cause of infertility		
Advanced patient age	213 (57.5%)	61 (63%)
Male factor	158 (42.7%)	42 (43.3%)
Tubal	87 (23.5%)	17 (17.5%)
Ovarian	46 (12.4%)	36 (37.1%)
Endometriosis	28 (7.6%)	4 (4.1%)
Unexplained	17 (4.7%)	1 (1%)
COS protocol		
Antagonist	289 (74.8%)	87 (87%)
Long agonist	97 (25.2%)	13 (13%)
Follicles	3.5 ± 1.6	3.1 ± 1.5
Estradiol level on hCG day	832.5 ± 165.7	748 ± 132.6

Patients may have more than one infertility factor

Table 2 Mean number of oocytes obtained after follicular aspiration and follicular flushing during ART cycles of poor ovarian responders with <5 oocytes at oocyte retrieval

	Oocytes	CI
Total	2.7 ± 1.1 (1–4)	(2.6; 2.8)
Aspirated	1.8 ± 1.2 (0–4)	(1.7; 2.0)
Flushed	0.9 ± 0.8 (0–4)	(0.8; 0.9)

Values are mean ± SD (range), *CI* confidence interval, Student's *t* test $p < 0.001$ comparing total *x* aspirated oocytes

Table 3 Mean number of oocytes obtained after follicular aspiration and follicular flushing during ART cycles of poor ovarian responders according to the Bologna criteria

	Oocytes	CI
Total	2.4 ± 1.1 (1–4)	(2.2; 2.6)
Aspirated	1.6 ± 1.1 (0–4)	(1.3; 1.8)
Flushed	0.9 ± 0.9 (0–3)	(0.7; 1.0)

Values are mean ± SD (range), *CI* confidence interval, Student's *t* test $p < 0.001$ comparing total *x* aspirated oocytes

Embryo transfer was performed on day 2 in 77.6% of cycles, on day 3 in 20.6%, and on day 5 in 1.8%. Overall pregnancy rate was 22%. A significant association was observed between pregnancy rate and the number of oocytes retrieved ($p = 0.002$), the number of MII oocytes ($p < 0.001$), and the number of embryos transferred ($p = 0.01$). Thus, the greater the number of obtained oocytes, MII oocytes, and transferred embryos, per cycle, the higher the pregnancy rate (Fig. 1).

We analysed 100 cycles of the Bologna criteria group (70 patients). A total of 309 follicles were aspirated (mean ± SD = 3.1 ± 1.5) and 242 oocytes recovered, with 156 (64.5%) being obtained by direct aspiration and 86 (35.5%) by follicular flushing. We found a significant difference between the total number of oocytes and the number of aspirated oocytes ($p < 0.001$) (Table 3). A total of 181 oocytes (74.8%) were MII, 60 (24.8%) were MI, and one (0.4%) was GV. Normal fertilization was confirmed in 72.6% (175) of the inseminated oocytes and development to cleavage stage embryos occurred in 71.4% (125), and 91.2% were considered of good quality.

Embryo transfer was performed on day 2 in 74.6% of cycles, on day 3 in 23.7%, and on day 5 in 1.7%. Overall pregnancy rate was 12.1%. We did not observe association between the number of oocytes retrieved, the number of MII, and the number of embryos with pregnancy rate in this group of patients.

Discussion

This retrospective study suggests that follicular flushing is an alternative to increase the number of retrieved oocytes and improve pregnancy rates of POR patients subjected to ART. All patients studied herein were POR, including the subgroup of patients matching the Bologna criteria, that is part of the POR group. The latter was analysed as a separate group since these criteria have been increasingly adopted in many centres.

The patients' mean age was >38 years. Therefore, it was expected that the most frequent cause of infertility was

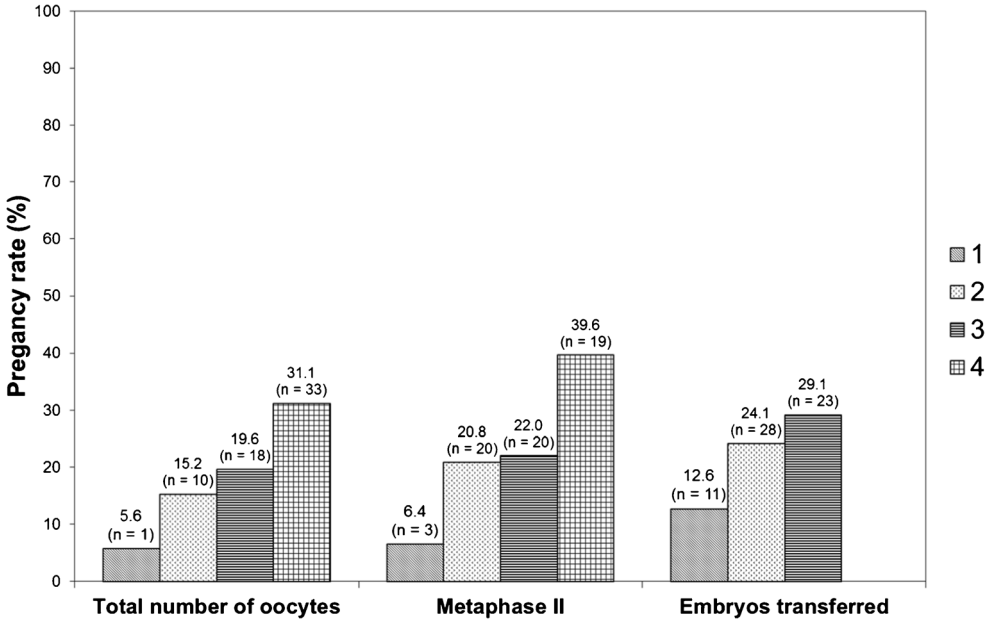

Fig. 1 Pregnancy rate according to the total number of oocytes, number of MII, and total number of embryos transferred in poor ovarian responder patients undergoing ART Mantel-Haenszel test

advanced maternal age since this factor is known to be the main cause of POR [8]. The antagonist protocol was used in most cycles because it has been suggested that it increases the number of follicles and oocytes [12]. The long protocol was used for young patients and for those with no previous history of POR.

We found that follicular flushing significantly contributed to the total number of oocytes retrieved in POR patients, regardless of whether they met the Bologna criteria or not. Moreover, the vast majority of the oocytes were at MII. Normal fertilization after ICSI was observed in 75%, and embryo development occurred in 75% of the fertilized oocytes, with >90% being considered of good quality. Our results contrast with those reported in two previous studies. Levens et al. (2009) did not observe an increase in the number of oocytes after follicular flushing in POR. This discrepancy might be explained by the limited number of patients included in their study ($n = 30$) and by the selection criteria used (4–8 follicles of ≥12 mm), which probably excluded patients who could potentially benefit most from follicular flushing. Mok-lin et al. (2013) also did not observe a difference in the number of oocyte retrieved after follicular flushing. Once again, we believe that the limited number of patients included in their study ($n = 50$) might explain the observed differences between their results and ours. The differences might also be explained by methodological differences as our study is retrospective and the others were prospective.

There was a significant impact of follicular flushing on the number of oocytes retrieved, the number of MII oocytes, and the number of embryos transferred with the pregnancy rate. As the increment in the number of oocytes was secondary to the use of follicular flushing, it is possible to assume that this technique might increase pregnancy rates. This finding contrast with previously published results [13, 14]. This discrepancy may be explained by differences in the methodology used in our study, as we did not compare the use of follicular flushing with a control group with no follicular flushing.

In the group of patients who matched the Bologna criteria, we did not observe an association between the number of oocytes retrieved and number of embryos transferred with pregnancy rate. The smaller number of patients in this subgroup may explain the lack of effect.

A possible flaw is the chance of the oocyte to be kept in tubing or needle form the first aspiration. However, as it is a limitation of the method and not from the study, it does not negatively impact the results as all procedures were performed the same way.

Our results suggest that follicular flushing might be a suitable alternative to increase the number of oocytes retrieved following ART and increase pregnancy rates in POR patients. However, additional studies are necessary to confirm its effectiveness since only few randomized studies with a limited number of subjects have been published and there is no ideal treatment of choice for these patients.

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