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ASSISTED REPRODUCTION TECHNOLOGIES

The association between multinucleated blastomeres and poor ovarian response under the Bologna criteria

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Abstract
Purpose To investigate the occurrence of multinucleated blastomeres (MNB) in poor ovarian response (POR) women defined under the Bologna criteria.
Methods This observational study was designed in a prospective controlled manner. Among 380 cases evaluated for eligibility, 102 women were found suitable and recruited; 51 with POR in accordance with the Bologna criteria defined as the study group and 51 with normal ovarian response defined as the control group.
Results Among the 51 women in each group, 8 and 2 did not achieve embryos in the study and control group, respectively ($P < 0.05$). The percentage of women that had at least one embryo with one MNB was significantly higher in the study as compared to the control group, corresponding to 49 and 29 %, respectively. The total number of embryos evaluated was 416; 167 in the study and 249 in the control groups. Among these embryos, the MNB rate was significantly higher in the study as compared to the control group, corresponding to 19 and 8 %, respectively.
Conclusions Blastomere multinuclearity is significantly more common in women and embryos of POR cases, defined under the Bologna criteria. Future studies are warranted to substantiate our observation that has the potential to be clinically implemented in this sub-group of women undergoing assisted reproductive technologies (ART) treatment.

Keywords Assisted reproductive technologies · Bologna criteria · Multinucleated blastomere · Ovarian reserve · Poor ovarian response

Capsule Blastomere multinuclearity is significantly more common in women and embryos of POR cases, defined under the Bologna criteria.

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Introduction

Blastomere nuclearity at 40–44 h post insemination/injection, when the embryo is at 2- to 4-cell stage, has been suggested as a morphological criterion to assess embryo quality. Normally, each cell at this stage should have a single nucleus while multinucleation indicates a breakdown of one or more cellular events. Although the precise etiology underlying multinucleated blastomeres (MNB) is still unclear, several mechanisms have been suggested. The first related to karyokinesis (nuclear replication) in the absence of cytokinesis. The second associated with errors in chromosome segregation and/or in packaging at mitosis through total disintegration of the mitotic spindle or consequently to an abnormal mitotic event [1, 2].

Multinucleated blastomeres have been correlated with impaired cleavage, increased fragmentation, and reduced in vitro developmental capacity [3–5], as well as low blastocyst formation rate [6–8] and reduced implantation potential [1, 4, 5, 9–12]. In addition, MNB has also been correlated to low live birth rate following assisted reproductive technologies (ART) treatment [8, 13]. As such, MNB was suggested to be included in the morphological criteria of top embryo scoring towards a

single-embryo transfer approach [14]. Blastomere nuclearity was also incorporated into evidence-based integrated morphology cleavage embryo score for implantation potential of day 2 embryos in an ART setting [15]. Furthermore, several reports have shown that MNB has a relation to chromosomal integrity of the 2–4 cell embryos suggesting that this morphological criterion could be used for embryo aneuploidy screening [4, 16, 17].

To the best of our knowledge, the topic of blastomere nuclearity and MNB in low ovarian reserve women undergoing ART treatment has not yet been explored. Decreasing pregnancy and live birth and increasing pregnancy loss rates in ovarian aging women have been primarily attributed to oocyte quality. This has been related to disordered regulatory mechanisms governing meiotic spindle formation and function leading to an increased prevalence of aneuploidy in aging oocyte.

Several approaches have been suggested to identify embryos with the highest implantation potential in an ART setting. One strategy is to extend the culture period and observe which embryos reach the blastocyst stage [18]. A second strategy is to subject all fertilized and divided oocytes to pre-implantation genetic screening and transfer only euploid embryos [19]. However, the implementation of these two strategies in low ovarian reserve women, with low oocyte and embryo yield, may be logistically intricate to accomplish. Examining the association between MNB and low ovarian reserve may provide information that might be clinically implemented in this sub-group of women undergoing ART treatment.

The introduction of the Bologna criteria for poor ovarian response (POR) as an early manifestation of low ovarian reserve and ovarian aging has offered a new platform to investigate this topic in a standardized mode [20, 21]. Therefore, our aim in this study was to prospectively examine the association between blastomere nuclearity and low ovarian reserve in an ART setting. Specifically, our objective was to investigate the occurrence of MNB in POR women employing the Bologna criteria.

Materials and methods

Patients

Between June 2012 and December 2013, infertile women qualified for IVF/ICSI-ET treatment that turned to Poriya Reproductive Medicine Unit were examined for eligibility and those that consented for participating in this study were recruited. Women enrolled were regularly menstruating with body mass index of 18–35 kg/m^2. All women had a normal uterine cavity determined by hysterosalpingography and/or hysteroscopy. Women with hypogonadotropic hypogonadism, uncontrolled diabetes mellitus, hyperprolactinemia or thyroid disease were excluded. As well, women with PCOS and severe endometriosis were excluded. Women included could participate in the study only once.

The study was approved by the Institutional Review Board (IRB). No additional interventions were employed to the routine clinical and laboratory standards for IVF/ICSI preparation and treatment. Informed written consent was obtained from all participating women.

Controlled ovarian hyperstimulation was chosen on a case to case basis according to standard clinical practice. The long/short GnRH agonist or the GnRH antagonist protocols were employed based on clinical judgment. Conventional IVF and/or intra-cytoplasmic sperm injection (ICSI) were performed according to the cause of infertility. A detailed description of sperm, oocyte, zygote, and embryo handling as well as embryo transfer and luteal phase supplementation in our unit have been described previously [22].

Randomization and outcome measures

The study was prospective, controlled, and observational in design. No additional intervention was added. The medical staff in the IVF and endocrine laboratories, as well as ultrasonographic performers was blinded to the drug regimen and conduct of the study. Since the study focused on low ovarian reserve, women defined to have POR were first recruited and defined as the study group. Poor ovarian response was defined in accordance with the Bologna criteria [20]. At least two of the following three criteria had to be present to establish the definition: (1) advanced maternal age (>40 years) or any other risk factor for POR, (2) a previous POR (≤3 oocytes with a conventional stimulation protocol), and (3) an abnormal ovarian reserve test (antral follicle count (AFC) <7 follicles). Risk factors for POR were employed as suggested earlier [23]. They included short menstrual cycle length (<25 days), previous ovarian cystectomy or oophorectomy, chronic smoking, unexplained infertility, previous chemotherapy and/or radiotherapy treatment, family history of premature menopause, X chromosome derangements, and fragile X mental retardation 1 pre-mutation. Parallel to the enrollment of women in the study group, infertile women with good ovarian reserve, presenting in line before or after the POR patients, were randomly recruited as controls. Recruitment and enrollment of women were performed by the first two investigators JSY and VYL.

The primary outcome measure was MNB occurrence among women in both groups of the study. Embryos in which one or more MNB observed were referred to as multinucleated embryos. The secondary outcome measure was MNB embryos among the total number of embryos achieved in each group.

Laboratory procedures

Blastomere nuclearity was looked for on a single observation, as routinely performed at our center, to minimize exposure to potentially deleterious effects to ambient temperature and pH conditions outside of the incubator. This evaluation was carried out by two senior embryologists with more than 20 years of experience each in the IVF field. In the majority of cases, blastomere nuclearity was looked for on day 2 following oocyte retrieval, 40–44 h following insemination/injection. In some cases, due to the weekend holiday, mono or multinuclear blastomeres were looked for on day 3, 64–68 h following insemination/injection. All observations were made with a Nikon Inverted Ti-U microscope (Nikon®, Tokyo, Japan) using Nikon advanced modulation contrast, (Nikon®, Tokyo, Japan) at ×300 magnification. Other laboratory procedures and morphological criteria were carried out as routinely performed at our IVF laboratory [22].

Ovarian reserve tests

Early follicular basal AFC (2–10 mm) and ovarian volume evaluation were performed in a natural cycle in all women before recruitment to the study as described previously [24]. Ovarian ultra-sonography was performed employing a transvaginal probe (5–9 MHz) (Voluson E-8; General Electric Medical System, Milwaukee, WI). As well, blood was drawn on the same day for serum basal FSH, LH, and E_2 levels as well as FSH/LH ratio evaluation. Ovarian reserve test chosen to fulfill the third criteria of the Bologna criteria was AFC.

Hormone assays

Sera obtained for basal FSH and LH measurements were analyzed by microparticle enzyme immunoassay (AxSYM®, Abbott, Abbott Park, IL, USA). The intra-assay and inter-assay coefficients of variation were <5 and <11 %, respectively, for FSH and <7 and <8 %, respectively, for LH. Serum E_2 and P levels were assayed by solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 2000, DPC, Los Angeles, CA, USA). The intra-assay and inter-assay coefficients of variation were <10 and <16 %, respectively, for E_2 and <18 and <22 %, respectively, for P. All hormonal assays were performed at the Poriya endocrinology laboratory.

Statistical analysis

We analyzed all data using the Software Package for Social Sciences (SPSS) for windows version 15.0 (SPSS Inc. 2006, Chicago, IL, USA). Descriptive procedure was used to evaluate patients' characteristics, and each variable is presented as mean ± SD. Independent two sample Student's t test and Z test for two independent proportions and logistic regression were used wherever appropriate. Normal distribution was analyzed prior to statistical tests using Wilk-Shapiro test. P value of <0.05 was considered as statistically significant.

Results

During the 19 months of the study phase, 380 cases were evaluated for inclusion in this study. One hundred and two women were found eligible, consented for the study and were prospectively recruited; 51 with POR in accordance with Bologna criteria defined as the study group and 51 with normal ovarian response defined as the control group (Fig. 1). The basic characteristics of these two groups are presented in Table 1. All parameters examined except age were similar between the two groups. The mean age of women was 37.7 ± 4.6 and 32.6 ± 6.4 in the study and control groups, respectively. The 5-year difference is explained by age inclusion in the first criterion of the Bologna criteria definition of POR.

Ovarian reserve tests are presented in Table 2. The study group had significantly inferior ovarian reserve as compared to the control group, evident by higher basal FSH level and FSH/LH ratio as well as lower AFC and ovarian volume.

The ovarian stimulation protocols, ovarian response, and IVF laboratory results are presented in Table 3. Although ovarian stimulation protocols were similar between the groups, the total FSH and LH dosages employed for stimulation were significantly higher in the study as compared to the control group. Albeit considerably amplified ovarian stimulation, ovarian response was significantly lower in the study as compared to the control group. This was evident by decreased E_2 level and lower number of ≥14 mm follicles on the day of hCG administration. Similarly, number of retrieved oocytes and fertilized oocytes were lower in the study group as compared to controls. Although the number of transferred embryos was similar between the two groups, the number of frozen embryos was lower in the study group.

Among 51 women in the study group, 8 did not achieve embryos while this was the case only in 2 women among the control group, this difference was statistically significant. The total number of embryos evaluated in this study was 416; 167 in the study and 249 in the control groups. The majority of cases (87 %) and embryos (90 %) were evaluated, for MNB detection, on day 2 post oocyte retrieval, while the minority was evaluated on day 3 post retrieval (13 and 10 %, respectively); this was performed due the weekend holiday. More cases and embryos were evaluated on day 3 in the study as compared to the control group (Table 4).

MNB occurrence (at least in one embryo) among women was significantly higher in the study as compared to the control group, corresponding to 49 and 29 %, respectively. In addition, among the total number of embryos evaluated in each group, the MNB rate was significantly higher in the

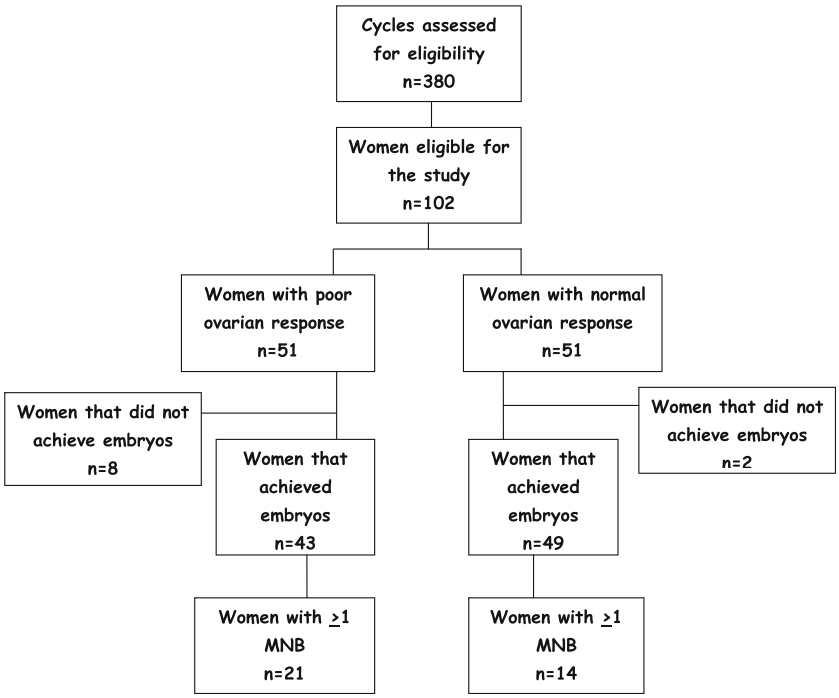

Fig. 1 Flow chart of the women recruited to the study

study as compared to the control group, corresponding to 19 and 8 %, respectively (Table 4).

To study the impact of young POR women on MNB occurrence, a sub-group analysis of our data was performed. Among the 51 women in the study group, 26 were <40 years of age. The mean age of this sub-group was 33.9 ± 3.5 years and not significantly different to the mean age of the control group (32.6 ± 6.4 years). Among these 26 women, 3 did not achieve embryos. The number of women with at least one MNB (≥1 blastomere) embryo was 12/23 (52 %) in this sub-group as compared to 14/49 (29 %) in the control group, and this difference was significant ($P = 0.026$). Furthermore, total number of embryos with MNB in this sub-group was 19/92 (21 %) as compared to 19/249 (8 %) in the control group, and this difference was significant ($P = 0.0007$).

Furthermore, to properly control for possible confounders especially age, multiple logistic regression analysis was performed to examine what factor significantly influenced MNB

Table 1 Patients' characteristics in the study and control groups

	Study group $n = 51$	Control group $n = 51$	P
Age (years)	37.7 ± 4.6	32.6 ± 6.4	0.001[a]
BMI (kg/m^2)	27.5 ± 0.3	25.3 ± 4.7	NS[a]
Chronic smoking	21.6 %	15.7 %	NS[b]
Duration of infertility (years)	6.1 ± 0.7	4.8 ± 4.0	NS[a]
Degree of infertility: primary infertility (%)	55.0	66.7	NS[b]
Secondary infertility (%)	29.4	25.4	NS[b]
Primary and secondary infertility (%)	15.6	7.9	NS[b]
Etiology of infertility: male (%)	33.5	37.2	NS[b]
Mechanical (%)	9.8	5.9	NS[b]
Endometriosis (%)	5.8	5.9	NS[b]
Unexplained (%)	35.2	23.6	NS[b]
Combined (%)	15.7	27.4	NS[b]

NS not significant

[a] *T* test for two independent samples

[b] Z test for two independent proportions

Table 2 Ovarian reserve tests in the study and control groups

	Study group $n=51$	Control group $n=51$	P^a
Antral follicle count	3.7 ± 2.2	13.7 ± 6.7	0.001
Total ovarian volume (cm^3)	14.6 ± 5.4	24.2 ± 14.6	0.005
Mean ovarian volume (cm^3)	7.3 ± 2.7	12.1 ± 7.3	0.005
Day 3 FSH (IU/L)	8.5 ± 3.2	6.8 ± 2.9	0.03
Day 3 LH (IU/L)	5.3 ± 0.7	6.2 ± 2.8	NS
Day 3 FSH/LH ratio	1.8 ± 0.3	1.2 ± 0.7	0.00004
Day 3 E_2 (pg/mL)	76 ± 2	78 ± 6	NS

NS not significant

[a] *T* test for two independent samples

occurrence taking into account age, infertility duration, BMI, basal FSH, FSH/LH ratio, AFC, ovarian volume, FSH and LH dosage employed during stimulation, and number of embryos achieved. Number of embryos achieved was the only significant factor that affected the occurrence of MNB in this setting supporting the association between MNB and POR.

Discussion

Our results clearly show that MNB occurrence in early cleaved embryos is significantly increased in POR cases. Women with POR, defined in accordance with Bologna criteria, had significantly higher rate of MNB as compared to controls, corresponding to 49 and 29 %, respectively. Moreover, among all embryos examined, the rate of multinucleation was significantly higher in the study as compared to the control group, corresponding to 19 and 8 %, respectively.

To the best of our knowledge, this is the first prospective study targeting the association between MNB and POR. Furthermore, the present work is the first to employ the Bologna criteria for POR to explore its relation to MNB occurrence. The significant differences between the study and control groups regarding ovarian reserve tests, ovarian response to COH, and IVF laboratory results support the two groups' comparability employing the Bologna criteria.

It may be argued that although the two compared groups had similar baseline characteristics, the POR group had significantly higher age compared to controls by about 5 years, negating a fair comparison. Age is a prerequisite of the Bologna first criterion, and this seems to explain age difference between the two groups. This may be as well one of the limitations of the criteria themselves that have been recently critically appraised [21].

Furthermore, the sub-group analysis performed strengthens our conclusion and support the notion that blastomere multinuclearity is significantly more common in women and embryos of POR cases, defined under the Bologna criteria. Young POR women under these criteria with a comparable age to the control group had higher MNB rates in their cycles as well as among their embryos in comparison to controls.

To properly control for confounding factors especially age, multivariate logistic regression analysis was performed to look for factors that had an impact on MNB

Table 3 Treatment protocols, ovarian response to controlled ovarian hyperstimulation, and IVF laboratory results in the study and control groups

	Study group $n=51$	Control group $n=51$	P
Treatment protocol: long GnRH agonist (%)	45	57	NS[b]
Short GnRH agonist (%)	33	29	NS[b]
GnRH antagonist (%)	22	14	NS[b]
Duration of stimulation (days)	10.4 ± 2.8	10.5 ± 2.5	NS[a]
Total FSH dose (IU)	3460 ± 1635	2744 ± 1619	0.03[a]
Total LH dose (IU)	2079 ± 1593	662 ± 1195	0.001[a]
Maximal E_2 level (pg/mL)	1693 ± 1022	2301 ± 1367	0.007
Progesterone level	0.91 ± 1.07	0.96 ± 0.46	NS[a]
Endometrial thickness (mm)	9.4 ± 2.5	10.1 ± 3.2	NS[a]
No. of ≥14 mm follicles	5.3 ± 4.2	9.3 ± 5.4	0.001[a]
No. of retrieved oocytes	3.4 ± 2.6	8.8 ± 5.8	0.001[a]
No. of fertilized oocytes	3.1 ± 2.8	4.4 ± 3.4	0.02[a]
No. of transferred embryos	1.7 ± 1.1	1.5 ± 1.0	NS[a]
No. of frozen embryos	1.06 ± 1.9	3.3 ± 3.8	0.01[a]

NS not significant

[a] *T* test for two independent samples

[b] Z test for two independent proportions

Table 4 Number of cases and embryos evaluated for multinucleated blastomeres (MNB) occurrence in the study and control groups

	Total	Study group	Control group	P^a
Number of women starting treatment	102	51	51	
Number of women with embryos	92	43 (84 %)	49 (96 %)	0.022
Women evaluated on day 2	80 (87 %)	34 (79 %)	46 (94 %)	0.017
Women evaluated on day 3	12 (13 %)	9 (21 %)	3 (4 %)	0.017
Women with MNB (≥1 blastomere)	35 (38 %)	21 (49 %)	14 (29 %)	0.022
Number of embryos	416	167	249	
Embryos evaluated on day 2	375 (90 %)	142 (85 %)	233 (93 %)	0.002
Embryos evaluated on day 3	41 (10 %)	25 (15 %)	16 (7 %)	0.002
Embryos with MNB on day 2	46 (11 %)	28 (20 %)	18 (7 %)	0.0003
Embryos with MNB on day 3	4 (10 %)	3 (12 %)	1 (6 %)	NS
Total embryos with MNB	50 (12 %)	31 (19 %)	19 (8 %)	0.0004

NS not significant

[a] Z test for two independent proportions

occurrence. Age was not found to affect MNB occurrence in our analysis. The same has been also reported by other investigators [14, 17, 25]. This may imply that MNB occurrence, similar to ovarian reserve studies (AFC and AMH), is a more appropriate "bio-marker" of ovarian reserve than age. However, this novel concept should be examined in a prospective-targeted study. The logistic regression analysis performed in our study showed that the number of embryos achieved is the only independent factor that significantly affect MNB occurrence, supporting the association between increased blastomere multinuclearity and POR, defined under the Bologna criteria.

Two previously published studies have suggested that MNB is a morphological phenomenon resulting from high ovarian stimulation and increased number of oocyte retrieval [5, 26]. These findings may also be supported by a later report showing that mild ovarian stimulation results in fewer oocytes and a decreased proportion of aneuploid and mosaic embryos in the normal responder population [27]. Although this might be true, it does not negate our findings. Both cited studies that were retrospectively designed did not target MNB occurrence in POR women. Our study was prospectively designed to specifically explore the occurrence of MNB in POR women, defined in accordance with the Bologna and did not target women with excessive ovarian response. It is possible that the MNB phenomenon is more prevalent in the opposite extremes of ovarian response; the excessive and the poor ovarian response. Future prospective-targeted studies are needed to substantiate our results and this concept.

Furthermore, several studies have shown that MNB is largely associated with an increased rate of aneuploidy and chromosomal abnormalities [1, 4, 16, 17, 27–31]. Oocyte chromosomal abnormality is believed to be the main cause of low fertilization, cleavage, and implantation rates in POR cases, demonstrating high incidence of human embryo incompetence leading to low pregnancy and high abortion rates in these women. Hence, it is not surprising for women with POR to have a higher rate of MNB than controls, as shown in our study.

Our study may have some limitations. The first related to MNB search for in a single observation. Since MNB is a dynamic phenomenon, one observation of embryonic growth can miss part of MNB detection [32, 33]. Conversely, early cleaved embryos have been shown to have the ability of self-correction from multinucleated to mononucleated during development, and this occurrence could be missed when employing a single inspection [34]. Nevertheless, since all embryos in both the study and control groups were similarly examined, this limitation does not seem to significantly alter our main finding that MNB is more prevalent in POR cases. Time lapse technology employment taking consecutive digital images of embryos at frequent time intervals, under stable culture environment, may increase the precision and sensitivity of current morphological evaluation. Prospective-targeted studies employing this novel technology to target blastomere nuclearity in early cleaving embryos among POR women may shed more light on this occurrence.

A second limitation could be attributed to the fact that more cases and embryos were examined on day 3 in the study group as compare to the control group (Table 4), and this may cause a bias when interpreting the data achieved. The literature is consistent with the fact that the rate of MNB is reduced on day 3 as compared to that on day 2 [5, 7]. This has been explained due to a self-correction phenomenon [34, 35] or due to the larger dimensions of day 2 blastomere size; their better optical accessibility and less cell overlap [5]. However, since the majority of cases and embryos (87 and 90 %, respectively) in the study were examined on day 2, it does not seem to have a major impact on our findings. More important, should all cases have been examined on day 2, this would have increased

even more the MNB occurrence in the study group, supporting further our findings.

In conclusion, in our prospective-targeted study, blastomere multinuclearity was shown to be significantly more common in women and in embryos of POR cases, defined in accordance with Bologna criteria, as compared to controls. Our findings may support the notion that MNB occurrence is related to chromosomal aneuploidy related to disordered regulatory mechanisms governing meiotic spindle function in aging oocytes. Time-lapse technology employment for MNB evaluation in POR cases could shed more light on this occurrence and provide information that may well be clinically implemented in this sub-group of women undergoing ART treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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