Spontaneous in vitro maturation and artificial activation of human germinal vesicle oocytes recovered from stimulated cycles

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Abstract

Purpose To define germinal vesicles (GV) by morphometric and morphologic examination and by chromatin compaction and to assess their spontaneous nuclear and cytoplasmic competence.

Materials 131 GV were cultured for 42.7 ± 2.4 h. Nuclear maturation was evaluated at four time points. Sixty-seven in vitro and twenty-five in vivo metaphase II (MII) were activated. Parthenotes with 2 PB and one pronucleus (NA) were studied for ploidy.

Results A total of 74.8% GV matured to MII: 55% at 21.4 ± 2.4 h and 47.3% in the following 24 h. Artificial activation induced NA in 79.2% of in vivo-MII and in 22.4% of in vitro-MII. All NA were haploid.

Conclusions GV spontaneously mature at the nuclear level. Their NA are haploid, but their cytoplasmic competence is

compromised. Variables were not found to be predictors of oocyte competence, probably due to our population being homogeneous with respect to most of the variables studied.

Keywords Germinal vesicle · Humans · Oocyte activation · Spontaneous in vitro maturation

Introduction

Fully grown oocytes mature to MII spontaneously when released from the follicular environment (>3 mm). In humans, in vitro maturation (IVM) occurs in 30–50% of oocytes recovered from non-stimulated cycles (1, 2) and in 60–80% of germinal vesicle (GV) oocytes removed from stimulated ovaries (3–8).

The heterogeneity of a cohort of follicles is maintained after hCG administration, which means that recovered oocytes vary in their capacity to mature, become fertilized and progress in their development. A cohort can include mature metaphase II (MII) oocytes, which usually have reproductive potential, and immature oocytes, which are discarded from IVF protocols. Immature oocytes also vary in their chromatin configuration (9–16), ability to progress to meiosis (9, 10, 17), and cytoplasmic and developmental competence (10, 13, 18, 19).

Firstly, we characterized our population of immature GV by morphometric and morphologic examination of different parameters (13, 14, 16) and established their chromatin condensation stage (16).

Secondly, we evaluated the nuclear and cytoplasmic competence of GV. Nuclear competence was defined as the ability to progress to MII during IVM. Cytoplasmic competence was defined as the ability of MII to become activated at fertilization, when the spermatozoon triggers a

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Capsule Immature oocytes recovered from stimulated cycles were morphologically and morphometrically defined. The variables obtained were studied as predictors of both the nuclear and cytoplasmic competence of in vitro-matured oocytes.

This work has been carried out at: University Institute IVI Valencia, Valencia, Spain.

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cascade of reactions that culminate in the resumption of meiosis and initial steps of early embryonic development. However, this oocyte activation can also be induced by physical and chemical stimuli that can be present in the absence of sperm (20–22). As fertilization is equivalent to activation (23–30), and since it can occur without the need for sperm, we applied an artificial oocyte activation protocol to diagnose the cytoplasmic competence of in vitro-matured oocytes. This protocol avoids the creation of embryos for purposes other than reproduction.

Finally, we aimed to correlate morphometric, morphologic and mathematically-predicted chromatin condensation-stage variables with the spontaneous nuclear and cytoplasmic competence of our population of oocytes.

Materials and methods

Institutional approval and informed consent

This work was approved by the ethics committee of the Instituto Universitario IVI (Valencia, Spain) and the Valencian regional government (Conselleria de Sanitat, Generalitat Valenciana). Written informed consent was obtained before oocyte collection.

Oocyte collection

Oocytes were obtained from 100 donors (27.7 ± 3.9 years) following controlled ovarian hyperstimulation and follicle puncture and were cultured for 2–4 h in 50 μ L human Tubal Fluid medium (hTF, IVI Barcelona, Spain) under standard culture conditions (37°C and 5% CO₂ in air). Cumulus cells were removed with pipettes following incubation in hyaluronidase (40 IU/mL, Sage, Tumbull, USA).

Cumulus-free oocytes were classified as mature (MII) or immature. The latter were defined as those with no polar body (PB) or nuclear structure (MI, metaphase I), or with a germinal vesicle structure with no PB in the perivitelline space (GV).

Twenty-five control oocytes were obtained from eight donors (25.6 ± 4.6 years).

Morphologic and morphometric features and predicted chromatin condensation stage of GV

Immediately after recovery, individual GV oocytes were IVM in hTF and morphologically examined at a magnification of 400 (Olympus, Barcelona, Spain). Three images of each oocyte were recorded. The image capture process lasted a maximum of one minute per oocyte. OCTAX software for image analysis was employed to measure oocyte size (cytoplasmic diameter, excluding zona pellucida, ZP), nuclear diameter and largest nucleolus-like body (NLB) diameter. These morphometric measurements are presented in μ m as either mean $\pm$ SD or 95% confidence interval values (CI95) and were recorded three times by two observers (16).

GV were also assessed morphologically to confirm the location of the nucleus (central or peripheral), appearance of nucleoplasm (smooth or rough) and presence of NLB, whose position (central or peripheral) and number were recorded. In addition, the continuity of the GV's nuclear envelope was studied and a progressive variation was established (16) as follows: well-defined (grade 1); irregular features (grade 2); and discontinuous envelope in some areas (grade 3).

In addition to the eight abovementioned variables, we predicted chromatin condensation stage by applying the following mathematical model (16):

$$\text{Model} = e^{[9.586 - 0.114 (\text{oocyte size}) + 1.013 (\text{nuclear envelope grade1}) + 2.099 (\text{nuclear envelope grade2}) + 1.500 (\text{nucleolus location}) + 1.648 (\text{nucleoplasm appearance})]}$$

The value that estimated the fit of the model (R^2 Nagelkerke) was 0.33. Predictive value was obtained by introducing all the variables of each of the oocytes analyzed and calculating the exponential function. If the value for a particular oocyte was ≥ 0.5 , it was considered that there was a high probability that its chromatin was condensed.

In vitro maturation

For IVM, 131 GV were cultured in 50 μ L hTF for 48 h in standard conditions. Oocytes were microscopically observed at four time points. Special attention was given to GVBD and 1 PB extrusion (MI and MII stage, respectively).

Oocyte activation

Sixty-seven in vitro-matured MII were artificially activated with calcium ionophore (A23187) and puromycin (30). In short, oocytes were exposed for 5 min to A23187 (8 mM, Sigma, Barcelona, Spain) and were subsequently cultured for 5 h in puromycin (10 μ g/mL; Sigma, Barcelona, Spain). After activation, eggs were cultured in hTF for 16–20 h and were then assessed for extrusion of the second polar body (2 PB) and number of pronuclei.

The presence of a pronucleus is the primary marker of activation (24). Nevertheless, there are five types of activation responses, each of which were assessed (31–

36): normal activation (NA), in which parthenotes possess the 1 PB and 2 PB and a single pronucleus; abnormal activation I (ANAI), in which parthenotes display the 1 PB and 2 PB plus 2 or more pronuclei; abnormal activation II (ANAI), in which the 1 PB and one or more pronuclei are present; incomplete activation (IA), in which the 1 PB and 2 PB are exhibited in the absence of a pronucleus; and oocytes that do not activate (MII, 1 PB and no pronucleus). Data of eggs showing signs of degeneration or atresia were not included in the activation rates.

To evaluate the activation status of the oocytes 25 in vivo-matured MII were, cultured and assessed as described above (control group).

Further cleavage was not assessed in mature or control parthenotes.

Chromosome analysis

Ploidy was determined in the NA of in vitro- and in vivo-matured oocytes. Other activation responses were not evaluated.

For chromosome determination, eggs were fixed using our technique (37, 38) with some modifications. In short, the ZP and two PB were removed to avoid chromosome signal interferences. Isolated parthenotes were incubated in a hypotonic solution and were then transferred onto a grease-free slide to spread and lyse cells.

Chromosomal analysis was performed by means of fluorescent in situ hybridisation (FISH) for five chromosomes—X, Y, 13, 18 and 21—using CEPX (Spectrum Blue), CEPY (Spectrum Gold), LSI13 (Spectrum Orange), CEP18 (Spectrum Aqua) and LSI21 (Spectrum Green).

Samples were analyzed with an Olympus AX-70 epifluorescence microscope (Olympus Optical Co., Hamburg, Germany) and images were recorded with an Olympus DP-50 camera.

Design and statistical analyses

To evaluate nuclear competence, we monitored the spontaneous IVM to MII of GV recovered from stimulated cycles.

The cytoplasmic competence of in vitro- and in vivo-matured oocytes was determined according to their ability to activate, measured by the presence of at least one pronucleus. Differences between the activation rate and type of response were analyzed using a Chi-square test.

To study the predictive value of morphometric and morphologic parameters and chromatin condensation stage in relation to oocyte competence, GV were grouped according to whether or not they reached the MII stage and formed pronuclei after artificial activation. Statistical differences between continuous variables were submitted to ANOVA analysis using Bonferroni's cor-

rection. Statistical analysis of discrete variables was performed using Chi-square tests. Differences were considered to be statistically significant when the *p*-value was lower than 0.05.

All statistical analyses were performed using SPSS 17.0.

Results

Description of the GV population

Morphologically, 92.4% of our GV exhibited a swollen, subcortical nucleus and 43.4% presented a nucleolus with a rough appearance. One NLB was detected in 126 of the 131 GV and was peripherally located in 74.0% of cases. All three nuclear envelope continuity grades were observed and followed a normal distribution: 29.8% grade 1, 59.5% grade 2, and 10.7% grade 3.

Morphometry revealed no significant differences between the mean diameter of the GV (average $106.5 \pm 3.8 \mu\text{m}$; $105.8\text{--}107.1 \mu\text{m}$, 95CI). Similarly, the mean diameter of their nuclei was $30.2 \pm 1.5 \mu\text{m}$ ($29.9\text{--}30.4 \mu\text{m}$, 95CI), and the largest NLB was $8.6 \pm 1.1 \mu\text{m}$ ($8.4\text{--}8.8 \mu\text{m}$, 95CI).

Morphological and morphometrical data compilation using the previously described mathematical model allowed the chromatin condensation state of 127 GV oocytes to be determined (16). The majority of GV oocytes displayed compacted chromatin (85.5% vs. extended 14.5%, $p < 0.05$).

Nuclear competence

Fifty-five percent of the original GV population progressed to MII during the first $21.4 \pm 2.4 \text{ h}$ of IVM (Time2; Table 1). Of the remaining immature oocytes ($n=55$), 47.3% progressed to MII within the following $24.1 \pm 0.8 \text{ h}$ (between Time2 and Time4). When the IVM period had concluded ($42.7 \pm 2.4 \text{ h}$), 74.8% GV were at MII and a significantly lower percentage of oocytes were found to have degenerated (12.2%) or remained arrested at immature stages (MI or GV: 13.0%; $P > 0.05$).

To assess nuclear competence, GV were retrospectively separated into those that matured to MII and those that did not and their morphological and morphometric data and predicted chromatin condensation stage were compared. None of the variables were found to be statistically relevant.

Cytoplasmic competence

Sixty-seven spontaneously in vitro-matured oocytes that were all healthy in appearance (assessed at Time2) and twenty-five in vivo-matured MII (controls) were assessed for cytoplasmic competence by artificial activation After

Table 1 Oocyte maturation stage (GV, MI and MII) at five time points (Time 0–Time 4) during in vitro maturation

Oocyte maturation stage:	Time 0	Time 1 (2.7±2.3) ^a	Time 2 (21.4±2.4) ^a	Time 3 (26.6±2.0) ^a	Time 4 (42.7±2.4) ^a	Total (42.7±2.4) ^a
GV (n,%)	131 (100)	83 (63.4)	13 (9.9)	12 (21.8)	11 (30.6)	11 (8.4)
MI (n,%)	0	48 (36.6)	42 (32.1)	24 (43.6)	6 (16.7)	6 (4.6)
MI (n,%)	0	0	72 (55.0)	15 (27.3)	11 (30.6)	98 (74.8)
Degenerated (n,%)	0	0	4 (3.0)	4 (7.3)	8 (22.2)	16 (12.2)
Total		131	131	55	36	131

^a mean±SD, hours of in vitro maturation at which point oocytes were assessed for maturation stage

GV Germinal Vesicle, MI Metaphase I, MII Metaphase II

No significant differences were observed between any of the entries

culture, it was observed that the 2 PB was extruded in all controls but in only 32.8% of IVM oocytes ($p<0.001$). Similarly, the frequency of the presence of at least one pronucleus varied according to the oocyte's origin; it was confirmed in almost all in vivo-matured oocytes (95.8%) but in significantly fewer IVM oocytes (59.7%; $p=0.002$; Table 2).

Type of activation also differed between in vitro- and in vivo-matured oocytes (Table 2). Most in vivo-matured eggs followed a normal activation display (NA, 79.2%), whose presence was significantly more frequent than among in vitro-matured oocytes (22.4%; $p<0.001$). Comparable ANAI rates were observed in in vivo- and in vitro-matured parthenotes (16.7% vs. 6.0%, respectively). Finally, ANAII was the most frequent response observed in parthenotes derived from in vitro-matured oocytes (31.3%), while this response was not observed in any of the in vivo-matured oocytes assessed ($p=0.004$, Table 2).

Once the cytoplasmic competence of each GV had been established, we retrospectively grouped and compared morphological and morphometric variables and predicted

condensation chromatin stage for the five types of activation response. Statistical analysis concluded that none of the variables were statistically relevant.

Ploidy determination

Seven in vivo- and seven in vitro-matured oocytes that became activated following the formation of a NA were employed for chromosome determination.

Reliable FISH results were finally obtained from 5 control eggs in which haploid endowment was confirmed.

The study of seven NA parthenotes acquired from in vitro matured oocytes indicated that 6 were haploid and one was haploid for chromosomes 13, 18 and X and nulisomic for chromosome 21.

Discussion

Almost 75% of the denuded GV oocytes recovered from stimulated cycles matured spontaneously to MII during a

Table 2 Responses of oocytes to artificial activation, according to their in vitro or in vivo maturation

MII oocyte origin	No activation (1 PB+0PN)	Incomplete activation (1 PB+2 PB+0PN)	Normal activation (1 PB+2 PB+1PN)	Abnormal activation I (1 PB+2 PB+>1PN)	Abnormal activation II (1 PB+≥1PN)
In vitro matured (n=67) ^a	24 (35.8)a	3 (4.5)a	15 (22.4)b	4 (6.0)a	21 (31.3)a
In vivo matured (n=25) ^b	0 (0.0)b	1 (4.2)a	19 (79.2)a	4 (16.7)a	0 (0.0)b

^a Five in vitro-matured MII oocytes were not included in the cytoplasmic competence assessment due to their unhealthy cytoplasmic appearance

^b One egg degenerated and was excluded from the activation rates

a,b within a column indicates statistical differences ($p<0.05$)

No activation (1 PB+0PN). MII stage oocytes that did not activated. The first polar body (1 PB) was present but there was no pronucleus (PN)

Incomplete activation (1 PB+2 PB+0PN). MIII stage oocyte with two polar bodies but no pronucleus

Normal activation (1 PB+2 PB+1PN). The expected oocyte state at fertilization; two polar bodies and one single maternal pronucleus

Abnormal activation I (1 PB+2 PB+>1PN). MII stage oocyte with an extruded second polar body (2 PB) and more than one pronucleus

Abnormal activation II (1 PB+>1PN). MII stage oocyte that did not extrude the second polar body and with more than one maternal pronucleus

period of 42.7 ± 2.4 h. In vitro-matured oocytes also responded to an artificial activation stimulus, but at a lower rate than their in vivo-matured counterparts. All normally activated in vitro- and in vivo-matured oocytes were confirmed to be haploid.

Morphological, morphometric and predicted chromatin condensation stage analysis of our GV population revealed that none of the variables assessed were predictors of nuclear or cytoplasmic competence. This may have been a result of our GV population not being sufficiently heterogeneous, as morphometric and morphological features were shared by 50–70% of the oocytes.

Experience has shown that GV released from antral follicles are capable of maturing to MII after a minimum of 22 h of culture (3–7, 39–42). 66.4% of our GV matured to MII within the first 26.6 ± 2.0 h of culture, while only 8.4% continued to mature after this period (total IVM 42.7 ± 2.4 h). The global nuclear maturation rate of our oocytes (almost 70%) was comparable to that reported previously for denuded GV recovered from stimulated cycles and matured under different culture conditions (80%: 3–7, 39–42), which suggests that nuclear maturation is a predetermined time-dependant phenomenon that occurs spontaneously in the majority of GV recovered from stimulated ovaries.

Cytoplasmic competence is described as the ability of arrested MII oocytes to resume meiosis, culminate this process with extrusion of the 2 PB, remodel and reprogramme the sperm head, form two parental pronuclei and continue development. This response is induced by the fertilizing spermatozoon, but can also be provoked by artificial parthenogenetic activation (20–22). Almost 80% of our artificially activated in vivo-matured oocytes formed one pronucleus, while 95% formed ≥ 1 PN after ICSI. This more or less equivalent response to fertilization and artificial activation (23, 25–30, 43) endorses artificial oocyte activation as a useful tool for the preclinical evaluation of experimental procedures (44). Indeed, our results show that parthenogenetic activation allows the cytoplasmic competence of in vitro-matured oocytes to be assessed. However, this model has its limitations, since it only allows us to assess the potential of a mature oocyte to reassume meiosis and to culminate the process in the extrusion of the second polar body. It does not allow us to determine the competence of an oocyte to remodel and reprogramme the sperm head or to form a male pronucleus and much less so its potential to continue in its development to the blastocyst stage. Nevertheless, our model would appear to constitute a reliable and convenient initial approach to assessing cytoplasmic competence, especially as it allows the creation of embryos for this purpose and the ethical problems associated with it to be avoided.

We obtained a high activation rate and an NA response similar to those reported previously (60–90%; (23, 24, 30,

43, 44). NA response demonstrates the ability of MII to culminate meiosis with a successful sister-chromatin segregation during PB2 extrusion and to subsequently form a haploid pronucleus. Given that this single pronucleus is the maternal genetic contribution to a hypothetical embryo, the NA response that we observed supports the application of our protocol in experimental procedures.

In the present work, we demonstrate the diagnostic value of parthenogenetic activation and show that human in vitro-matured GV oocytes from stimulated ovaries respond to artificial stimuli, though at a lower rate than in vivo-matured oocytes, which is contrary to that stated in earlier reports (3–7, 39–42). The variations observed in activation rate and response with respect to the maturation process may reflect the deterioration in oocyte quality that accompanies IVM, probably due to abnormalities arising during cytoplasmic maturation, unsuitable IVM conditions and/or intrinsic or induced defects in developmental ability and/or aging.

Most of the in vitro-matured oocytes selected for activation were at MII when they were monitored (21.4 ± 2.4 h). This means that they had reached MII at an earlier point of culture and, consequently, were activated at varying post-MII aging. These time points are unknown, as no exact recordings of complete nuclear maturation were performed. Therefore, future studies of real-time maturation and artificial activation are required to clarify this aspect and optimize the cytoplasmic competence of in vitro-matured oocytes.

Conclusions

Our GV population was relatively homogeneous in terms of the morphological, morphometric and chromatin condensation stage variables studied. Naturally, these variables were not shown to be predictors of oocyte competence. Nevertheless, we have observed that immature oocytes from stimulated ovaries are capable of maturing spontaneously at the nuclear level, though their cytoplasmic competence can be compromised. In this sense, future work is necessary to evaluate and optimize this approach. We also demonstrate the efficiency of our artificial activation protocol for assessing the cytoplasmic competence of oocytes, which allows the creation of human embryos for purposes other than reproduction to be avoided.

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