Does the prolonged *in vitro* maturation of human oocytes influence the aneuploidy type?

Ali podaljšano zorenje v pogojih *in vitro* vpliva na vrsto anevploidij pri človeških jajčnih celicah?

Lidija Križančić Bombek, Veljko Vlaisavljević, Borut Kovačič

Oddelek za reproduktivno medicino in ginekološko endokrinologijo, Univerzitetni klinični center Maribor, Ljubljanska ul. 5, 2000 Maribor

Korespondenca/ Correspondence:
dr. Lidija Križančić Bombek, univ. dipl. biol., Oddelek za reproduktivno medicino in ginekološko endokrinologijo, Univerzitetni klinični center Maribor, Ljubljanska ul. 5, 2000 Maribor
e-mail: lidija.krizancic@uni-mb.si,
tel.: +386 23305857,
fax: +386 23305853

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Izvleček

Izhodišča: Oogeneza obsega mnogo mehanizmov, ki omogočijo zorenje citoplazme in jedra jajčne celice v točno določenem časovnem sosledju. Le pri primerno velikih in razvitih jajčnih clicah se ob ustreznih endokrinih in parakrinih signalih profaza prve mejotske delitve (*angl.* germinal vesicle stage, GV) nadaljuje do metafaze druge mejotske delitve (MII). Tudi v pogojih *in vitro* so jajčne celice sposobne, da spontano dozorijo, kar je povzročilo, da so se razvile tehnike oploditve z biomedicinsko pomočjo (OBMP). Eden ključnih dejavnikov za uspešnost postopka OBMP je pridobitev večjega števila zrelih jajčnih celic, kar dosežemo s hormonskim spodbujanjem jajčnikov. Kljub temu z aspiracijo jajčnih mešičkov pogosto dobimo nezrele jajčne celice, ki sicer lahko dozorijo v pogojih *in vitro* (*angl. in vitro* maturation, IVM), a je njihova klinična uporabnost še vedno vprašljiva zaradi pomanjkanja raziskav o njihovi morfološki in genetski kakovosti. V naši raziskavi smo proučevali vpliv trajanja IVM na vrsto in pogostost kromosomskih nepravilnosti pri jajčnih celicah iz hormonsko spodbujenih ciklov, ki so bile v času aspiracije v profazi prve mejotske delitve.

Metode: V raziskavi je sodelovalo 86 bolnic, starih 22–43 let, ki so darovale 198 nezrelih jajčnih celic. Rast jajčnih mešičkov smo spodbujali s kombinacijo agonista gonadoliberina in rekombinantnega folitropina. Ko je vodilni mešiček dosegel velikost 18 mm, so bolnice prejele rekombinantni človeški horijev gonadotropin, sledila pa je aspiracija jajčnih mešičkov.

Po odstranitvi granuloznih in kumulusnih celic (cumulus oophorus) smo jajčne celice z vidnim zarodnim mešičkom (GV) gojili v atmosferi s 6-odstotnim CO_2 in 95-odstotno relativno vlažnostjo pri 37 °C. Po 24 urah smo fiksirali vse dozorele jajčne celice in njihova prva polarna telesa (PT1) (Skupina A), po nadaljnjih 12 urah pa še preostale dozorele jajčne celice in njihova prva polarna telesa (PT1) (Skupina B).

Sledilo je označevanje kromosomov 13, 16, 18, 21 in 22 po navodilu proizvajalca sonde za fluorescentno hibridizacijo *in situ* (*angl.* fluorescent *in situ* hybridization, FISH). Pri normalnih jajčnih celicah smo dobili po 2 signala za vsak kromosom (2 kromatidi) v jajčni celici in po 2 signala za vsak kromosom v njenem prvem polarnem telesu. V jajčni celici s hipohaploidijo je bil za določeni kromosom 1 signal, v njenem prvem polarnem telesu pa 3 (obratno pri hiperhaploidni jajčni celici). Pri nulisomiji v jajčni celici signala za določen kromosom nismo videli, v njenem prvem polarnem telesu pa so bili 4 signali (obratno pri disomiji).

V skupini A in skupini B smo izračunali odstotek anevploidnih jajčnih celic ter pogostost različnih vrst kromosomskih nepravilnosti. Skupini smo primerjali s testom hi-kvadrat, pri čemer smo upoštevali mejo značilne statistične razlike $p < 0,05$.

Rezultati: Dozorelo je 65,2 % vseh jajčnih celic (40,9 % v skupini A in 24,2 % v skupini B). Pri 102 jajčnih celicah smo dobili komplementarno genetsko sliko za jajčno celico in njeno prvo polarno telo (skupina A: N=65 in skupina B: N = 37). Deleža anevploidnih jajčnih celic se med skupinama nista statistično razlikovala (35,4 % v skupini A in 48,6 % v skupni B), a bi ob večjem številu analiziranih jajčnih celic pričakovali statistično več anevploidnih jajčnih celic v skupini B.

Nadaljnja analiza je potekala na ravni kromosomov (325 kromosomov v skupini A in 185 kromosomov v skupini B), saj so imele mnoge celice na različnih kromosomih različne vrste anevploidij. Oba mehanizma nastanka anevploidij, tako predčasno ločevanje kromatid kot nerazdvajanje kromosomov, sta bila pogostejša v skupini B. V tej skupini smo opazili predvsem statistično

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pogostejše pojavljanje hiperhaploidij in disomij v primerjavi s skupino A (12,43 % vs. 5,54 %; p < 0,01). Podaljšano zorenje je najbolj vplivalo na povečanje pogostosti anevploidij kromosomov 18 in 22.

Zaključki: Pogostejše predčasno ločevanje kromatid in nerazdvajanje kromosomov pri jajčnih celicah, ki so zorele daljši čas, lahko kaže, da postopek IVM negativno vpliva na njihovo genetsko kakovost. Obstaja pa tudi možnost, da nekatere jajčne celice že v profazi prve mejotske delitve (GV) nosijo genetske nepravilnosti, ki jim otežujejo normalno zoritev. To se lahko odraža s počasnejšim zorenjem ali s pogostejšim pojavom anevploidij.

Abstract

Objective: In many stimulated infertility treatment cycles some oocytes are collected immature and can be matured *in vitro* (IVM). However, their safe clinical use is questionable because of their apparently low morphologic and genetic quality. We investigated the influence of the IVM duration on the type and frequency of chromosome abnormalities in germinal vesicle stage (GV) oocytes from stimulated cycles.

Design: *In-vitro* maturation of GV oocytes (from stimulated cycles) for 24 (Group-A) or 36 (Group-B) hours and subsequent fluorescent *in situ* hybridization analysis (FISH) of chromosomes 13, 16, 18, 21 and 22.

Results: After maturation, chromosomes were undoubtedly analyzable in 102 oocyte-first polar body pairs. Aneuploidy rates in both groups did not differ statistically. However, within Group-B a significantly higher rate of hyperhaploidy over hypohaploidy was observed. Also, there was a significantly higher frequency of disomy than nullisomy in both groups, and the aneuploidy rate of chromosomes 18 and 22 was significantly increased in Group-B.

Conclusion: The observed preferential excess over the loss of genetic material and the increase in chromosome non-disjunction in the oocytes from Group-B, indicate a negative influence of prolonged IVM on chromosome segregation or conversely, suggests that GV oocytes which attain maturity later, possess more intrinsic abnormalities that result in aneuploidy.

Introduction

The first meiotic arrest occurs early in prenatal life when the oocyte proceeds through the first stages of meiosis and stops at the diplotene stage of the first prophase. The maintenance of the meiotic arrest involves many complex biochemical mechanisms[1] and interactions with cumulus cells that communicate with the oocyte through cell junctions.[2-4] Human oocytes acquire meiotic competence in a sequential fashion during oogenesis, when growth, RNA synthesis, redistribution of organelles, and chromatin remodeling take place.[5,6] Only the fully-grown oocytes with specific chromatin configurations undergo germinal vesicle breakdown (GVBD) and proceed through meiosis I to metaphase II.[7,8]

It was observed early that mammalian oocytes undergo spontaneous maturation upon removal from the follicle[9-10] and *in vitro* fertilization (IVF) techniques began to develop. Today, the success of an infertility treatment depends partially on obtaining as many mature oocytes as possible, thus improving chances of fertilization. However, in many stimulated IVF cycles some or even all oocytes are collected immature (germinal vesicle–GV or meiosis I–MI stage).[11-14]

Recently, an *in vitro* maturation (IVM) method was developed in which pre-ovulatory immature oocytes are aspirated and matured *in vitro.*[15-17] In stimulated cycles with only immature cells retrieved, this method could be used to get mature oocytes. However, there are only few reports on clinical results after embryo transfer of *in vitro* matured oocytes from stimulated cycles.[18-22]

The safe routine clinical use of IVM oocytes from stimulated cycles is still under debate, since the quality of such oocytes seems low. Some investigations have been done concerning structural characteristics of chromatin and meiotic spindle,[23,24] however little is known about chromosomal abnormalities of IVM oocytes.[25-27]

In our research we investigated the aneuploidy rate of the oocytes from stimulated cycles, which attain maturity *in vitro,* and from this point of view represent a risk of conceiving an affected child. Furthermore,

we tested the influence of IVM duration on the aneuploidy rate of chromosomes 13, 16, 18, 21 and 22, which belong to those most commonly found in spontaneous abortions.[28]

Material and methods

One hundred and ninety eight immature germinal vesicle stage oocytes (GV-oocytes), collected from 86 patients undergoing assisted reproduction treatment for different infertility factors, were subjected to *in vitro* maturation (IVM). Patients with polycystic ovaries, patients with the majority or all immature oocytes, and patients with more than three previous unsuccessful infertility treatment cycles were excluded from the study. The mean age of patients was 32.2 ± 4.8 years (range 22–43 years) in Group A and 31.4 ± 5.2 years (range 23–40 years) in Group B (Mann-Whitney rank sum test, not significant). The study was approved by the National Medical Ethics Committee and the informed consent was obtained from each patient. All patients were pretreated with oral contraceptives with a combination of 0.03 mg ethynilestradiol and 0.075 mg gestodene (Femoden, Scherring AG) for minimum 18 days to maximum 35 days. Last contraceptive pill was taken 5 days before administration of follitropine was started. Controlled ovarian stimulation was performed as described earlier.[29]

After the ultrasound-guided ovarian aspiration, the oocytes were collected in Flushing medium (MediCult, Jyllinge, Denmark) and denuded of cumulus oophorus enzymatically (Hyadase, MediCult) and mechanically with a narrow pipette. Mature oocytes were used in standard IVF/ICSI treatment procedures, whereas oocytes with visible germinal vesicle (GV-oocytes) were transferred into separate drops of BlastAssist-M1 medium (MediCult) under paraffin oil (MediCult), and cultured at 37 °C in an atmosphere of 6 % CO_2 and 95 % relative humidity. Following the 24-hour IVM, GV-oocytes were inspected for the presence of the first polar body (PB1). If the PB1 was present, the oocyte and its corresponding polar body were fixed (Group A). Otherwise, the oocytes (GV or M I) were left in culture for another 12 hours. After 36-hours of IVM, additional mature oocytes were fixed comprising Group B. Those oocytes that failed to complete maturation within 36 hours were excluded from the study.

The matured oocytes were fixed as described by Dozortsev and McGinnis[30] and our group.[29] Chromosomes 13, 16, 18, 21 and 22 were stained and analyzed according to the protocol recommended by the manufacturer of the probe MultiVysion™ PB (Vysis, Downers Grove, IL, USA). Microscope images were observed under x1000 magnification with Axioplan 2 Imaging microscope (Carl Zeiss AG, Germany) and computer software CytoVision 2.81 (Applied Imaging, San Jose, USA). In a normal oocyte or first polar body, two FISH signals for each tested chromosome were visible (one signal for each chromatid). In a hypohaploid or hyperhaploid oocyte, there were one or three signals, respectively, with the reverse situation in their corresponding PB1. Oocytes with zero or four visible signals (with the reverse situation in their corresponding PB1) were classified as being nullisomic or disomic, respectively.

Frequencies of aneuploid and normal oocytes were calculated in both groups. The data were also analyzed with respect to the total number of individual chromosomes analyzed. The two groups were compared by chi-square test with the difference being statistically significant at $p < 0.05$. The average age of patients in both groups was tested by Mann-Whitney rank sum test, and the age of euploid and aneuploid oocytes within both groups was tested by one-way ANOVA test.

Results

The overall maturation rate after 36 hours of *in vitro* culture was 65.2 %, with 81 (40.9 %) oocytes from Group A and 48 (24.2 %) from Group B. In Group A, 65 oocyte-PB1 pairs (80.3 %) were undoubtedly analyzable and showed exactly complementary chromosome statuses. In 11.1 % of preparations, PB1 (7 cases), oocyte (1 case) or both (1 case) were lost during fixation,

Table 1: Aneuploidy rate of oocytes matured *in vitro* for 24 or 36 hours.

A	Group A (24h)	Group B (36h)
No.(%) of analysable oocyte-PB1 pairs	65	37
No. (%) of euploid oocytes	42 (64.6) [a]	19 (51.4) [a]
No. (%) of aneuploid oocytes	23 (35.4) [a,b]	18 (48.6) [a,b]
B		
Oocytes with single aneuploidy	15 (23.1)	12 (32.4)
Oocytes with double aneuploidies	6 (9.2)	2 (5.4)
Oocytes with multiple aneuploidies	2 (3.1) [c]	4 (10.8) [c]

Percentages (in brackets) are calculated as the number of aneuploid oocytes per 65 or 37 analyzable oocyte-PB1 pairs in Group A or Group B, respectively.
[a] *average age of oocytes: GroupA-euploid: 32.5 ± 4.8 years; GroupA-aneuploid: 32.3 ± 4.9 years; GroupB-euploid: 31.5 ± 5.5 years; GroupB-aneuploid: 31.3 ± 5.1 years; no signifficant difference (one-way ANOVA on ranks test)*
[b] *not significant (chi-square test)*
[c] *numbers too small for statistical analysis*

whereas in 7 preparations (8.6 %) signals for individual chromosomes were missing or were FISH errors with the PB1 not confirming the oocyte diagnosis. Similarly, 37 oocyte-PB1 pairs (77.1 %) from Group B had complementary chromosome statuses, in 9 preparations (18.7 %) the oocyte or PB1 were lost and 2 were FISH errors (4.2 %). The aneuploidy frequency in the 6 oocytes from the three patients older than 40 years was not different from other oocytes.

Only the complementary oocyte-PB1 pairs were subjected to further analysis. The aneuploidy rate was 35.4 % in Group A and 48.6 % in Group B. Although the difference between both groups was not statistically significant (Table 1A), a trend toward increasing aneuploidy frequency with IVM duration was observed. Furthermore, we expect that with an increased number of analyzed oocytes, the difference would become statistically significant. Similarly, a higher frequency of oocytes with multiple aneuploidies (three or more aneuploid chromosomes out of 5 analyzed) was observed in Group B compared to Group A (10.8 % vs. 3.1 %; Table 1B), but the number is too small for the difference to be statistically significant.

Further analysis was conducted on individual chromosomes ($65 \times 5 = 325$ chromosomes in Group A and $37 \times 5 = 185$ chromosomes in Group B) since each aneuploidy type can affect any chromosome and different aneuploidy types can be present in the same oocyte. Many oocytes carried double and multiple aneuploidies, which were frequently of different types.

Unbalanced premature chromatid separation resulted in significantly more hyperhaploid (4.32 %) than hypohaploid chromosomes (0.54 %) within Group B, whereas the total number of unbalanced premature chromatid separations in Group B did not differ from Group A (Table 2A). The predominant aneuploidy mechanism in Group B was chromosome non-disjunction (12.43 % in Group B vs. 5.84 % in Group A; $p < 0.01$), resulting in the presence or absence of a whole chromosome (two chromatids) in the oocyte. The frequency of nullisomy as well as disomy increased with the lengthening of the maturation period. However, only the increase in disomy frequency was statistically significant.

Within Group B, the excess of genetic material in the oocyte, including both hyperhaploidy and disomy, was significantly more frequent than the loss of genetic material from the oocyte, represented by both hypohaploidy and nullisomy (12.43 % vs. 4.86 %; $p < 0.01$; Table 2B). Within Group A, the frequency of excess genetic material in oocytes was slightly, but not significantly, higher than the loss of genetic material. When comparing the two groups, the excess

of genetic material was more frequent in Group B ($p < 0.01$).

We also analyzed the aneuploidy rate of each individual chromosome in both groups (Table 2C). The results show a significant increase in the aneuploidy rate of chromosomes 18 and 22 in Group B, suggesting a strong influence of the prolonged IVM on the meiotic segregation of these chromosomes. On the contrary, the remaining three chromosomes were not significantly affected.

Discussion

According to our results, oocytes spending longer time in IVM conditions have higher aneuploidy rate (48.6 % in group B vs. 35.4 % in group A). Nevertheless, the difference was not statistically significant, most probably due to a small number of analyzed oocytes. According to previous reports, detrimental effects of prolonged *in vitro* culture on oocytes[16,25,31] together with an increased rate of spindle and chromosome abnormalities are expected.[24,29,32] In our study, we found an increased frequency of aneuploid chromosomes in oocytes matured *in vitro* for a longer period of time (Group B), suggesting a negative impact of the IVM on the meiotic chromosome segregation. Furthermore, the aneuploidy analysis of individual chromosomes showed that chromosomes 18 and 22 were the most affected by the prolonged IVM, which is also consistent with data obtained by other authors.[33,34]

On the other hand, it is important to emphasize that the immature oocytes from our stimulated cycles were exposed to hCG *in vivo*, but did not respond to this maturation stimulus. From this perspective, it could be suspected that the inability to mature *in vivo* originates in intrinsic defects of these oocytes. To support this idea, Combelles et al.[8] reported that human oocytes are capable of maturing *in vitro*, but are generally unable to maintain second meiotic arrest and proceed to interphase within 24 hours following PB1 extrusion. This phenomenon was related to specific cell cycle deficiencies including restricted microtubule acetylation, changes in histone H3 phosphorylation and consequently chromatin decondenzation.[8] However, the more frequent aneuploidies may also be attributed to the exposure to FSH during the stimulation protocol, which might facilitate the maturation of oocytes with pre-existing chromosomal errors.[35]

Past and present research on oocyte aneuploidy suggests that a high proportion of fresh and IVM oocytes carry chromosomal aberrations. Unfortunately, for ethical reasons, we were not able to obtain a proper control group for our experiments, which would be donated fresh mature oocytes. Neither did we use non-fertilized oocytes due to problems with discerning the male or female origin of chromosomes, or their polar bodies, because they were frequently fragmented at the time of fertilization assessment. Therefore, we compared our results with the existing research data on different genetic material from oocytes. Our data are quite well comparable to aneuploidy rate of *in vivo* matured aspirated oocytes reported by Kuliev et al.,[36] who reported 46.7 % of tested PB1 having aneuploidies of either chromosome 13, 16, 18, 21 or 22. Similarly, the analysis of all 23 chromosomes in fresh donated oocytes by Sandalinas et al.[37] revealed 29 %–56 % aneuploidy rate depending on the patients' age. If we were to draw conclusions from these comparisons, we could speculate that IVM oocytes are of the similar quality as *in vivo* matured ones. Although, we found that 35.4 % (Group A) and 48.6 % (Group B) of IVM oocytes posses at least one type of aneuploidy on chromosomes 13, 16, 18, 21 or 22; a higher aneuploidy rate would be expect, if more chromosomes had been tested, because also those chromosomes, which were not tested in our study, can carry different aberrations and add to the final aneuploidy rate. Indeed, a higher aneuploidy rate of 60 % in donated and IVM oocytes was recently reported by Requena et al.[34] who analyzed chromosomes 13, 15, 16, 18, 21, 22 and sex chromosomes.

Li et al.[24] have demonstrated that *in vitro* matured oocytes from unstimulated cycles have a significantly higher frequency of abnormalities in microtubule configurations of the meiotic spindles than *in vivo* matured oocytes (43,7 % vs. 13.6 %, respectively).

Table 2: Frequency of different aneuploidy types.

A		Group A (24h)	Group B (36h)
Unbalanced premature chromatid separation	Hypohaploidy	8 (2.46)	1 (0.54) [a]
	Hyperhaploidy	6 (1.85)	8 (4.32) [a]
	Total no. of premature chromatid separations	14 (4.31)	9 (4.86) [c]
Chromosome nondisjunction	Nullisomy	7 (2.15)	8 (4.32)
	Disomy	12 (3.69) [b]	15 (8.11) [b]
	Total no. of chr. nondisjunctions	19 (5.84) [d]	23 (12.43) [c, d]
B			
Loss of genetic material from the oocyte (hypohaploidy + nullisomy)		15 (4.62)	9 (4.86) [f]
Excess of genetic material in the oocyte (hyperhaploidy + disomy)		18 (5.54) [e]	23 (12.43) [e, f]
C			
Chromosome aneuploidy rate	chr.13	2.15	3.24
	chr.16	4.00	2.70
	chr.18	0.62 [g]	3.78 [g]
	chr.21	2.15	2.16
	chr.22	1.23 [h]	5.41 [h]

Percentages (in brackets) are calculated as the number of aneuploidy cases of the specific chromosome per total number of 325 (65x5) or 185 (37x5) chromosomes analyzed in Group A and Group B, respectively. If one oocyte had more than one type of aneuploidy, it was counted once for each aneuploidy type.
[a–b] *$p < 0.05$ (chi-square test)*
[c–h] *$p < 0.01$ (chi-square test)*
chr. = chromosome

They also reported a signficantly higher rate of chromosome configuration abnormalities in meiotic spindles of IVM oocytes in comparison to *in vivo* matured oocytes (33.3 % vs. 9.1 %). Similar data were obtained by Wang and Keffe[32] who reported that 48.1 % of GV or M I oocytes from stimulated cycles that matured *in vitro,* had abnormal spindle and chromosome configurations in addition to 14.8 % of those with apparently normal spindle and dispersed chromosomes. From this data, it can be speculated that the high incidence of aneuploidies found in our study can be partially attributed to the misalignment of chromosomes in the meiotic spindle and disturbed chromosome segregation. As shown before, the majority of aneuploidies of mature oocytes originate in the first meiotic division either because of balanced or unbalanced premature separation of chromatids, or because of whole chromosome nondisjunction.[38-41] After having analyzed the rate of different aneuploidy types, a significantly higher frequency of extra chromatids compared to missing chromatids was found in Group B, but not in Group A. This correlates with the observations of Kuliev et al.[36,42] who found more hypohaploid than hyperhaploid PB1 in patients of advanced maternal age, suggesting the reverse situation in oocytes. In addition, extra chromosomes were more frequent than missing chromosomes in both groups. The observed excess of hyperhaploid and diploid oocytes implies that in cases of chromatid and chromosome mall-segregation, excess genetic material preferably remains in the oocyte. This finding supports the existence

of a speculative intrinsic oocyte mechanism, which prevents extrusion of an extra genetic material into the PB1 as already suggested by Kuliev et al.[36]

The overall maturation rate of 65.2 % in the present study is in agreement with previously reported 34 %–84 % of *in vitro* matured GV oocytes in different cultivation media and protocols.[21,34,43-45] The result suggests that a reasonable proportion of oocytes can achieve nuclear maturity even in a medium, which is routinely used for embryo cultivation. However, if available, specific IVM media supplemented with hormones and growth factors are recommended, since they provide better milieu for the developing oocytes.[45,46]

In conclusion, there seems to be a trend towards an increase in aneuploidy rate with the lengthening of the IVM period. However, to irrefutably demonstrate this, a larger number of oocytes is needed. In this study, we showed an increase in the frequency of oocytes with the excess of genetic material (hyperhaploidy + disomy) in Group B, which points to a negative influence of prolonged IVM on normal chromosome segregation. Alternatively, our results might indicate that GV oocytes with more pre-existing intrinsic abnormalities, which later result in aneuploidy, are less competent in achieving maturity (e.g. they attain maturity later). If the IVM oocytes are to be used in a clinical practice as a supplement to IVF treatment, it should be kept in mind that their genetic quality is still questionable.

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