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Cytogenetic aberrations in primary cell cultures of the ovarian surface epithelium.

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Key words: cell cultures, chromosome aberrations, chromosomal instability, ovarian epithelium, polyploidy.

Abstract. Our objective was to determine the presence of chromosomal abnormalities in primary cultures of ovarian surface epithelial cells in women of different ages with no history of cancer. Throughout conventional cytogenetic techniques, we analyzed chromosome spreads of cultured ovarian epithelial cells from 10 donors who were 50 or more years old (B) and 16 controls between 20 and 49 years old (A), belonging to the mestizo population in Bogota DC, Colombia. Of the 26 cultures that were analyzed in passage 1, 61.5% had an abnormal chromosome complement (62.5% in A, and 60% in B). Abnormalities included polyploidies, endoduplications and monosomies. Deletions in chromosomes 3 and 11 were found in just one metaphase. None of the samples showed weaknesses or breakpoints. After transforming and applying the exact student’s t-test for variance heterogeneity, we found significant differences in the frequency of metaphases, that were higher in A than in B (p=0.05), and in the frequency of polyploidies, which were higher in B than in A (p=0.044). Through the application of the Mann-Whitney test, we determined that the frequency of endoduplications was higher in A than in B (p=0.126), without reaching significant differences. There were no significant differences in the frequency of monosomies. The level of significance was set at p ≤ 0.05. Taking into account that polyploidization is a marker of chromosomal instability and that the risk of cancer arising from the ovarian surface epithelium augments substantially after menopause, the increase in the frequency of age-associated polyploidies could be used as a predictor of ovarian cancer in women from an ethnically homogeneous population as the mestizo one in Bogota DC.
**Aberraciones citogenéticas en cultivos primarios del epitelio superficial del ovario.**

*Invest Clin 2010; 51(4): 541 - 551*

**Palabras clave:** cultivos celulares, aberraciones cromosómicas, inestabilidad cromosómica, epitelio ovárico, poliploidía.

**Resumen.** El objetivo del presente trabajo fue determinar la presencia de anormalidades cromosómicas en cultivos primarios de células del epitelio superficial ovárico en mujeres de diferentes edades, sin antecedentes de cáncer. Mediante técnicas de citogenética convencional fueron analizados extendidos de células epiteliales ováricas histológicamente normales, provenientes de cultivos primarios de 10 donantes de 50 o más años (B) y de 16 donantes entre 20 y 49 años que se utilizaron como grupo control (A), pertenecientes a la población mestiza de Bogotá DC, Colombia. De 26 cultivos examinados en pase 1, 61,5% presentó complemento cromosómico anormal, 62,5% en A y 60% en B. Las anomalías numéricas halladas, todas en mosaico, incluyeron poliploidías, endoduplicaciones y monosomías. En una única célula en metafase de un cultivo, se presentaron delecciones en los cromosomas 3 y 11. Ninguna muestra presentó fragilidades o roturas. Previa aplicación de transformaciones con la prueba exacta t-student para varianzas heterogéneas, se encontraron diferencias significativas en la frecuencia de células con metafase normal, mayor en A que en B (p=0,05) y en la de poliploidías, mayor en B que en A (p=0,044). Con la prueba exacta de Mann-Whitney se determinó que la frecuencia de endoduplicaciones en A fue mayor que en B (p=0,126), sin alcanzar diferencias significativas y que no hubo diferencias significativas en la frecuencia de monosomías. El nivel de significación fue p ≤ 0,05. Si se tiene en cuenta que la poliploidización es un marcador de inestabilidad cromosómica y, que además, el riesgo de aparición de cáncer derivado del epitelio superficial del ovario aumenta sustancialmente después de la menopausia, el incremento en la frecuencia de poliploidías asociado con la edad podría ser utilizado como predictor de cáncer ovárico en mujeres de una población étnicamente homogénea como la población mestiza de Bogotá DC.

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**INTRODUCTION**

Almost 80% of ovarian carcinomas arise in the surface epithelium (OSE) of this organ and are responsible for more than half of the deaths related to gynaecologic diseases (1-4). In particular, the annual incidence of ovarian cancer in Colombia is 8.6 per 100000 women of which 7% occur in premenopausal and 30% in postmenopausal women, according to the Registro Poblacional de Cáncer from Cali, Colombia (5). Signs of genetic instability in this tissue are in close association with malignant transformation (6). These changes include chromosomal abnormalities (7,8), as deletions in region 6q15-q21 of chromosome 6, pericentri-
version of chromosome 9 inv(9)(p11q13), loss of chromosomes 3p, 6p, 6q, 9q, 11p, 13q, 17p13 y 17q, trisomies 12, 7 and 8, monosomies X (A, B, C, D, E, F, G, H) and polyploidies (with randomly positioned chromosomes) (9-16), which usually precede chromosomal abnormalities. Other changes, such as translocations between chromosomes 6 and 14 t(6;14)(q21;q24) and between chromosomes X and 11 t(X;11)(q23;q23), or point mutations that provoke oncogene activation, such as kras (17) and inactivation of tumour suppressor genes such as p53 (18) and brca1 (19) among others, have been also related to the origin of this pathology. Tetraploidies in particular, correspond to the replication of both genomic content and number of centrosomes which emerge as the result of defective cytokinesis or chromosome segregation during mitosis.

It has been possible to identify a spectrum of chromosomal abnormalities, both non-random and highly complex, in primary ovarian carcinomas. Although they are not characterized by pathognomonic rearrangements, they contain the either erroneous arrangements or structural abnormalities such as the ones mentioned previously (21, 22).

In this context, and considering that the risk of cancer arising from OSE increases in perimenopausal women, karyotypes of cultured ovarian epithelial cells from 26 donors aged 20-63 years old, which had gone through total or partial oophorectomy due to benign conditions, such as ovarian cysts, adnexal torsion, or salpingo-oophorectomy due to ectopic pregnancy or benign adnexal masses. The tissues were histologically normal.

Donors belonged to an ethnically homogeneous cohort of Colombian mestizo women who were taken to surgical procedures in five hospitals, both public and private, in Bogota DC, during 2007 and 2008. The sample was stratified into two age ranges, as follows:
- A. - <50 years
- B. - ≥50 years

To set this stratification, we took into account that the risk of cancer arising from OSE increases significantly with age, especially after menopause.

**Primary cultures**

To separate the surface epithelium from stroma, each tissue sample was processed through enzymatic disaggregation with 0.5% Dispase II (Roche Molecular Biochemicals) and then seeded in the media mix MCDB105 (Sigma)/M199 (Gibco) supplemented with 10% fetal bovine serum (FBS). After reaching 90-95% confluence in culture flasks of 25 cm², cells were detached through the enzymatic action of trypsin/PBS solution and 0.25% EDTA for 60 seconds.

After resuspending in mixed media and FBS, cells were placed again in culture flasks. The flasks contents were filled up to 3 mL with culture medium and 10% FBS and incubated at 37°C and 5% CO₂. Cells were serially subcultured until 70-80% of them had acquired a senescent phenotype (large cells of mesenchymal appearance hardly confluent) for approximately 4-5 passages.

**Karyotype**

After breaking up cells in passage 1 with trypsin/EDTA and centrifuging, we
added medium and resuspended the pellet. The cell suspension was deposited on plates in petri dishes, covered with 15 mL MEM (Sigma) supplemented with 10% FBS, and incubated during 24 hours at 37°C. When a monolayer appeared, we added 150 µL of colchicine for 2.5 hours and then an hypotonic solution (0.075 M KCl) for 10 minutes. This step was previously standard-ized in order to obtain the correct cell dispersion without undesirable presence of lysis or metaphases mixes. Then, we pre-fixed the spreads for 10 minutes and fixed them for 50 minutes more with Carnoy.

Differential staining

Chromosome preparations were aged during 2 hours at 82°C and incubated in 2xSSC buffer for 30 min at 60°C. Afterwards, trypsinization was carried out by immersion in trypsin-water 1:1 solution for 5 seconds and stained with Giemsa for 10 minutes. Slides were sealed to be examined under the microscope.

Chromosome analysis

For the lecture, we selected 279 cells in metaphase with appropriated morphologic characteristics, dispersion, and chromosomal bands. In total, three to four spreads from each donor were analyzed. To examine polyploidy with randomly positioned chromosomes, we only took into account chromosomes with similar condensation level, well-closed and well-banded chromatides, in order to avoid any confusion between true polyploidy and the lysed metaphases formed as a result of the hypotonic treatment.

Charaterization

We determined the expression of cytokeratin 18 through immunocytochemical techniques. Cytokeratin 18 is a cytoskeletal fibrillar protein present in the nucleus, nucleolus, and cytoplasm of simple epithelium cells. The cell spreads on slides, previously fixed with Carnoy, were stained with monoclonal antibody anticytokeratin 18 conjugated with fluorescent isothiocyanate (FITC) (Sigma) and incubated in a moist chamber for 12 hours at 37°C and 5% CO₂. After washing with PBS, green signals were visualized by fluorescence spectroscopy using a FITC/Rhodamine/DAPI triple filter.

Statistics

By applying Kolmogorov-Smirnov (Lilliefors) and Shapiro Wilk tests for normality, we evaluated the following variables: total number of metaphase cells, number and frequency of normal and abnormal metaphase cells and frequency of polyploidy, endoduplications and monosomies per group in passage 1. The Levene test was applied to determine variance homogeneity. In case there were no normal distributions, square root transformations were made. In order to establish whether there were differences between means, we used the student’s t-test for independent groups with homogeneous or heterogeneous variances. When assumptions were not met, we applied the exact non-parametric Mann-Whitney test. The level of significance was set at p ≤ 0.05.

RESULTS

Of the 26 cultures examined at passage 1, 61.5% had an abnormal chromosome complement (62.5% in A and 60% in B). These complements included numerical abnormalities, all mosaicisms, such as polyploidy (with randomly positioned chromosomes) (Fig. 1), endoduplications (Fig. 2) and monosomies. There were structural abnormalities only in a single metaphase cell that were evidenced as deletions in chromosomes 3 and 11. None of the samples showed weaknesses or breakpoints (Table I).
The exact nonparametric Mann-Whitney test showed that in A, both total number and normal metaphase cells were significantly higher than in B (p=0.043 and p=0.028, respectively). The frequency of abnormal metaphase cells in B was almost significantly higher than in A (p=0.139) (Table II).

After applying square root transformation, the exact student’s t-test adjusted for heterogeneous variances showed that frequency of normal metaphase cells in A was higher than in B (p=0.005) and that there was a significant difference in the frequency of polyploidies between groups, higher in B than in A (p=0.044) (Table II).

Through the analysis of Fig. 3, we determined that both mean and median frequencies of abnormal metaphase cells were higher in B, whereas in A there were frequencies equal to zero.

The exact Mann-Whitney test established that frequency of A cells with endoduplications was higher than in B.
TABLE 1
CHROMOSOME ABNORMALITIES AND THEIR FREQUENCIES AT PASSAGE 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Chromosome complement</th>
<th>% normal metaphases /total metaphases</th>
<th>% abnormal metaphases /total metaphases</th>
<th>% polyploidies /total metaphases</th>
<th>% polyploidies /abnormal metaphases</th>
<th>% endo duplications /total metaphases</th>
<th>% endo duplications /abnormal metaphases</th>
<th>% monosomies /total metaphases</th>
<th>% monosomies /abnormal metaphases</th>
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<td>34</td>
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<td>11.1</td>
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<td>0</td>
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<td>0</td>
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<td>100</td>
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<td>11.5</td>
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<tr>
<td>Age group</td>
<td>% normal metaphases / total metaphases</td>
<td>% abnormal metaphases / total metaphases</td>
<td>% polyploidies / total metaphases</td>
<td>% polyploidies / abnormal metaphases</td>
<td>% endo duplications / total metaphases</td>
<td>% endo duplications / abnormal metaphases</td>
<td>% endo duplications / total metaphases</td>
<td>% endo duplications / abnormal metaphases</td>
<td>% monosomies / total metaphases</td>
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<tr>
<td>&lt; 50 n=17</td>
<td>Mean 0.05 0.139 0.044 0.095 0.126 0.199 0.654 0.654</td>
<td>Mean 0.1893 18.933 17.438 42.089 11.945 40.121 2.6948 24.254</td>
<td>Mean 0.818 18.228 10.190 28.088 7.384 24.853 0.654 5.88</td>
<td>Mean 0.05 0.139 0.044 0.095 0.126 0.199 0.654 0.654</td>
<td>Mean 0.1893 18.933 17.438 42.089 11.945 40.121 2.6948 24.254</td>
<td>Mean 0.818 18.228 10.190 28.088 7.384 24.853 0.654 5.88</td>
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<tr>
<td>≥ 50 n=9</td>
<td>Mean 0.564 43.589 41.569 55.556 2.020 11.111 0 0</td>
<td>Mean 0.459 45.937 47.565 52.704 6.060 33.333 0 0</td>
<td>Mean 0.818 18.182 15.789 100 0 0 0 0</td>
<td>Mean 0.564 43.589 41.569 55.556 2.020 11.111 0 0</td>
<td>Mean 0.459 45.937 47.565 52.704 6.060 33.333 0 0</td>
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<tr>
<td>Total</td>
<td>Mean 0.730 27.007 21.052 37.596 5.527 20.096 0.427 3.85</td>
<td>Mean 0.325 32.497 33.917 46.906 10.480 37.818 2.179 19.612</td>
<td>Mean 0.818 18.182 15.789 100 0 0 0 0</td>
<td>Mean 0.730 27.007 21.052 37.596 5.527 20.096 0.427 3.85</td>
<td>Mean 0.325 32.497 33.917 46.906 10.480 37.818 2.179 19.612</td>
<td>Mean 0.818 18.182 15.789 100 0 0 0 0</td>
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DISCUSSION

Our study showed that both total metaphase and normal metaphase cells were higher in cultured tissues from younger donors. In the elderly ones, although there was proliferation, responsiveness to culture conditions was slower. This reduction in replicative capacity and the ageing associated changes previously reported in literature (23-25), may influence the occurrence of the chromosomal abnormalities described herein. Regarding this, in many cases, polyploidies appeared since the first passage. The obvious question is whether this type of numerical abnormality was present in vivo, or instead, it was induced by the stress culture. Before seeding cells, karyotype is not examined, since this requires that metaphase cells are analyzed and it is only possible when cells grow in culture. Taking into account that polyploidies occur during cell division and also that the ovarian surface epithelium is subject of a high turnover rate at the site of rupture caused by ovulation, it is possible that such abnormalities have appeared before the sampling. This idea would gain more strength if we consider that the karyotype analysis was performed in the first passage, in a relatively similar way as the one used for tissue removal. However, if this did occur, it would confirm that the probability of polyploidies in ovarian surface epithelium in vivo is greater in women over 50 years. Moreover, the abnormalities were not found as isolated cases, fact supporting the idea that culture stress did not induce the appearance of such abnormalities.

Furthermore, there was not a possibility of confusing true polyploidies with the metaphases resulting after the hypotonic treatment. These ones are easily distinguishable because their chromosomes exhibit variable condensation levels and morphology, in contrast to the true polyploidies.

The occurrence of polyploidies is associated with chromosomal instability and therefore with a higher risk of malignant transformation. It is also related to the appearance of the senescent state. This can be explained on the basis of DNA replication in absence of mitosis, which is a usual behavior in senescence (26-29). For this reason, we consider polyploidization as a predictor of cancer risk and also as a senescence marker (30, 31). Our results suggest that cultured cells of the ovarian surface epithelium became senescent in an early stage and more frequently in tissues from donors over the age of 50. Regarding the adaptive advantages that this represents at cellular level, It has been proposed that at early ages, cell senescence acts as a tumor suppressor mechanism (32, 33), while in advanced ages it promotes tumorigenesis (33, 34).

As a cancer risk predictor, polyploidization seems to be a crucial stage in the evolution of aneuploidies and carcino-
genesis (36). In particular, tetraploidies correspond to chromosome stages that have been associated with development of aneuploidies and chromosome instability (37, 38), but also appear in germ and somatic cells. Germ tetraploidies, which are very rare, are incompatible with life and emerge as the result of a defective mitosis during early embryonic development (39). Tetraploidies are frequent in human somatic cells, particularly in tissues with higher capacity of self-renewal, such as liver, placenta, certain hematopoietic cells (40-42) and others who normally do not proliferate, as cardiac muscle. In this case, it is believed that polyploidization acts as an adaptive mechanism against DNA damage and oxidative stress.

In our study, six of 26 total metaphase cells (23.1%) and six of the 16 abnormal ones (37.5%) were tetraploidies. It is important to note that this abnormality appears in the early malignant transformation (32, 47, 50) and it is common in precancerous and cancerous stages of some types of tissues (45) as the ovarian surface epithelium (6,46), where its presence is significantly correlated with an increased risk of tumor progression (47-50).

Because of the association between chromosome abnormalities and carcinogenesis, a higher probability to develop polyploidies in ovarian surface epithelium in the elderly should have an impact on the clinical field, in specific situations such as:

- Identification of pre-neoplastic changes in the normal ovary versus the cancerous one.
- Evaluation of karyotype changes in the ovarian surface epithelium adjacent to carcinoma of this tissue.
- Comparison of chromosomal abnormalities in the ovaries of monozygotic twins, one of which has had ovarian cancer.

- Comparison between karyotype of ovaries prophylactically removed in women at risk of developing cancer and those of both Colombian and world general population (51).

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