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MICRONUCLEUS ASSAY IN PRENEOPLASTIC INJURIES OF CERVICAL CANCER

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ABSTRACT

Introduction: Cervical cancer ranks fourth among the ten leading global causes of death, preinvasive lesions and invasions of the cervix are a public health problem. In the state of San Luis Potosi, Mexico, it is common to see women between 20 and 30 years old. Is necessary found biomarkers in preneoplastic injuries, for that reason we evaluate the genotoxic risks (observed as DNA damages) by Micronucleus (MN) Assay in lymphocytes of peripheral blood and exfoliated epithelial cervical cells.

Aim: To identify the occurrence of MN in preneoplastic lesions and to find the correlation between MN and preneoplastic lesions at the time of diagnosis (zero time), 3, 6 and 12 months after the treatment in lymphocytes and exfoliated epithelial cervical cells.

Materials and Methods: A total of 160 females were included in the study and visual examination of the cervix was done. Based on the examination: four groups were formed: A-normal cervix (n=40); B-CIN I (cervical intraepithelial neoplasia I; n=40); C-CIN II (cervical intraepithelial neoplasia II, n=40); and D-CIN III (cervical intraepithelial neoplasia III, n=40). Blood and cervix cells sample was collected after getting the informed consent. After 3, 6 and 12 months of treatment, samples of epithelium and blood were also taken. Then were cultured, with RPMI and DMEM-F-12 supplemented and incubated at 37 °C and 5% CO_2 for five days in 24-well plates. Slides were prepared from the pellet, were fixed in methanol and glacial acetic acid fixative 1:1 and stained with PAS-PAP stain modified. The presence of MN was confirmed under oil immersion (100 X), observations were recorded. Two hundred cells were recorded in each patient from the slides prepared and the incidence of micronucleus was recorded with IMAGE PRO PLUS software and the collected data was subjected to ANOVA test with GraphPad Prism v.7.

Results: ANOVA test showed us significative differences between the mean MN count of healthy individuals and the mean of preneoplastic lesion stage, CIN I, CIN II, and CIN III at zero time. The same behavior was observed at longer time, twelve months. The Group B (CIN I), C (CIN II) and D (CIN III) ases had significant MN count at three, six and twelve months compared with healthy individuals (Group A). Nevertheles we not found significative differences when compared lymphocytes and epithelial cervical cells. In other results, was obtained that on average in lymphocytes there was a presence of 3.5 % of micronuclei and epithelial cells of 4.5% in healthy women, CIN I 21 vs 22%, CIN II 39 vs 36 % and CIN III 66.5 vs 68%. It was shown that there is no statistically significant difference between counts in lymphocytes and epithelial cells. Therefore the lymphocytes is the better option for MN assay.

Conclusion: A statistically significant MN count was seen in the different stages of preneoplastic lesion of cervix. MN assay is an easy, non-invasive, cost-effective method and can be used as a screening test for a large population. The micronucleus test would be a useful tool in the diagnosis of cancer supported by other diagnostic techniques of genotoxic damage.

INTRODUCTION

Cancer arises from one single cell. The transformation from normal cell into a tumors cell is a multistage process, typically a progression from a precancerous lesion to malignant tumors [1]. These changes are the result of the interaction between a person's genetic factors and three categories of external agents, including: physical carcinogens, chemical carcinogens, and biological carcinogens, such as infections from certain viruses [2]. The cancer cervix accounts for 10.60% amongst all cancers in Mexico and is the second most common cancer [3].

MN originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [4]. The assay is being applied successfully for biomonitoring of in vivo genotoxin exposure, in vitro genotoxicity testing and in diverse research fields such as nutrigenomics and pharmacogenomics as well as a predictor of normal tissue and tumor radiation sensitivity and cancer risk. The procedure can take up to 5 days to complete. The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MN in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided binucleated cells (BN), which are the cells that can express MN. MN,

therefore, provide a convenient and reliable index of both chromosome breakage and chromosome loss. In the CBMN assay, once-divided binucleated cells are recognized by their appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring (Concanavalin A, Con-A) assembly required for the completion of cytokinesis [5-7]. Restricting scoring of MN in BN cells prevents confounding effects caused by suboptimal or altered cell division kinetics, which is a major variable in micronucleus (MN) assay protocols that do not distinguish between non-dividing cells that cannot express MN and dividing cells that can. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells. Scoring of MN can be performed easily and on different cell types relevant for human biomonitoring, in these case with lymphocyte and exfoliated epithelial cervical cells, with additional in vitro cultivation step.

MN observed in exfoliated cells is not induced when the cells are at the epithelial surface, but when they are in the basal layer [8]. The aim of the study was to identify the occurrence of MN in cases with normal cervix, cases with growth in the cervix and to identify the occurrence of MN in different stages of preneoplasic lesions. By using this technique cancer can be detected at an early stage.

MATERIAL AND METHODS

1.Overview of study design

This study was performed in the laboratory of Biochemistry and Microbiology in University of San Luis Potosi, from June 2016 to June 2018. An ethical approval was obtained from the Institutional Ethical Committee. An informed written consent was taken from each patient. A total number of 160 samples of patients were collected (healthy, CIN I, II, III). The patients do not suffering from other pathologies.

2. Cells and culture conditions

Cultured peripheral blood cells (PBCs) and exfoliated epithelial cervical cells(EECCs)

The cells have been isolated from healthy individuals once informed consent obtained or patients with diagnosis CIN I, CIN II and CIN III. The was collected fresh blood by venipuncture into vacutainer blood tubes with anticoagulant, and then gently overlay diluted blood onto Histopaque using ratio 1:3, being very careful not to disturb the interface. Removed the leucocyte layer located at the interface of Histopaque and diluted plasma into a fresh tube, using a sterile,plugged, pasteur pipette, taking care not remove too much Histopaque. The lymphocytes cells were cultured in (RPMI) containing 10% serum fetal bovine and 1% penicillin/streptomycin, in 37 °C at 5% CO_2. Each cervical scraping was performed with an endocervical brush (cytobrush) that was placed inside a polypropylene tube with 2 mL of PreservCyt. The exfoliated epithielial cervical cells (EECCs) was washing with PBS 1X, were cultured with Dulbeccos Modified Eagle's media DMEM-F-12 supplemented and incubated at 37 °C and 5% CO_2 in 24-well plates.

Cell growth curve and MN assay

A count of lymphocytes and epithelial cells was performed at 0 hours, at 24 hours, and a stimulus was given with 3 µL of Concanavalin A (5 ug/mL), at 72 hours and stimulation was given with 1.8 µL of Cytochalasin B (6 ug/mL) at 96 hours and harvested the cells. From the lymphocytes and epithelial cells preparations were made on precleaned slides. Up to two to three slides were made and were allowed to air dry.

After air drying, the slides were kept in absolute methanol for 20 minutes for the fixation of cells. The slides were kept in PAS-PAP stain. Stained slides were mounted with cover slip.

The presence of MN was confirmed under oil immersion (100 X), observations were recorded and tabulated. Two hundred cells were recorded in each patient from the slides prepared and the incidence of micronucleus was recorded with IMAGE PRO PLUS software and the collected data was subjected to ANOVA test.

RESULTS

Cultivation of peripheral blood cells (PBCs) and exfoliated epithelial cervical cells(EECCs) in 24-well plates can be utilized for genotoxicity testing in terms of MN-induction

Following this protocol, we have enhanced the method to cultivate lymphocytes and epithelial cells in 24-well plates for genotoxicity testing in terms of MN-induction. The slides showed lymphocytes and epithelial cells with micronucleus (Figure 1). At time of harvesting, a slight difference in the MN induction frequency of lymphocytes (3.5%) and epithelial cells (4.5%) was found in healthy women (P-value***<0.0004, A-B). The same thing happened for NIC I (21 vs 22%; P-value****<0.0001, C-D) and NIC II (39 vs 36%, P-value****<0.0001E-F). But, NIC III (66.5 vs 68%) do not showed significative differences (P-value, ns, ns= not significative, 0.3678, G-H; Figure 4). It was shown that there is not difference between counts in lymphocytes and epithelial cells. Therefore both cell types can be used for the induction of MN, with better results for lymphocytes.

ANOVA test showed us significative differences between the mean MN count of healthy individuals and the mean of preneoplastic lesion stage, CIN I, CIN II, and CINIII at zero time The same behavior was observed at longer time, twelve months. The Group B (CIN I), C (CIN II) and D (CIN III) cases had significant MN count at three, six and twelve months compared with healthy individuals (Group A, Figure 5).

Cell growth curve

Regarding the cell growth curve, the lymphocytes presented a better response to the stimuli applied and we consider them a viable option in the identification and counting of micronuclei. In the induction of micronuclei, at 24, 72 and 96 hours, no significant differences were found, both for healthy individuals and with lesions (Figure 3).

But the epithelial cells did show significant differences, in each of the sampling times (Figure 4).

DISCUSSION

Cervical cancer ranks fourth among the ten leading global causes of death, preinvasive lesions and invasions of the cervix are a public health problem. The cancer cervix accounts for 10.60% amongst all cancers in Mexico and is the second most common cancer [3].

Genomic instability refers to an increased tendency of alterations in the genome during the life cycle of cells. It is a major driving force for tumorigenesis [9]. Genomic instabilities can be assessed by MN assay. MN originate from chromosome fragments, complete chromosomes lagging behind in anaphase or amplified genome regions, which are excluded from the nucleus by a process called nuclear budding [4]. We found significantly elevated frequency of Micronucleated (MNd) cells in all patients with CIN III, irrespective of the when the sample was taken [10].

MN frequency and staging of preneoplastic lesion was statistically significant compared to control group. Genetic damage in cervix cancer patients was greater as compared to that in the control (healthy) group [11].

The mean MN score was most significant in CIN I, CIN II and CIN III. MN frequency in our study was significantly higher in women with cervical lesions than in woman showing cervical cancer, according Ambroise and collaborators [12-13].

This study indicates that the MN assay in cervical cells, may prove beneficial to perform in screening programmes of cervix cancer, given the non-invasive method of sample collection, easier availability and the rapidity of the assay [14-15].

This assay can be done as a screening test among the women in perimenopausal age group in large population by using automated MN assay. Females in the peri menopausal age group were not ready to volunteer for the cervical screening programmes or failed to come for the follow up. So, the early detection of the cancer cervix will be delayed.

Is much easier the collection of blood samples what cervical scrapings. The test can find applications in pilot screening programmes. In Mexico, an inexpensive easy method like MN assay can be suggested as a part of routine gynaecological examination. The early detection and appropriate treatment measures can eventually assist in bringing down the morbidity and mortality.

CONCLUSION

A statistically significant MN count was seen in the different stages of preneoplastic lesion of cervix. MN assay is an easy, non-invasive, cost-effective method and can be used as a screening test for a large population. The micronucleus test would be a useful tool in the diagnosis of cancer supported by other diagnostic techniques of genotoxic damage.
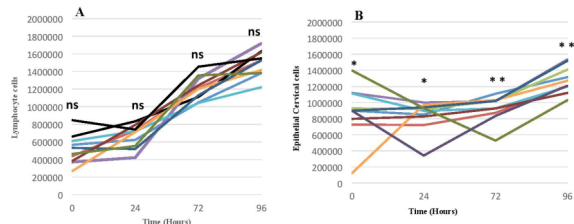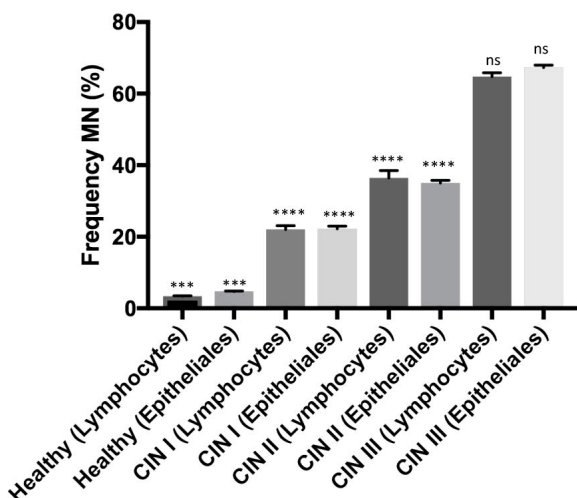
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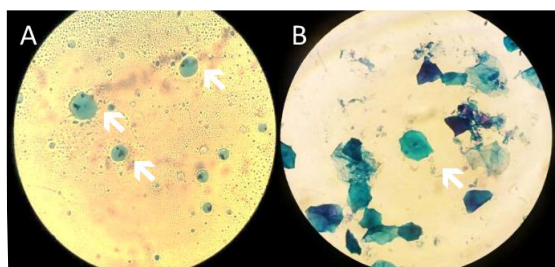
Figure 1. Preparations showing lymphocytes and epithelial cells with micronucleus (White arrows). (A) lymphocytes x100; (B) Exfoliates Epithelial cervical cell x100.
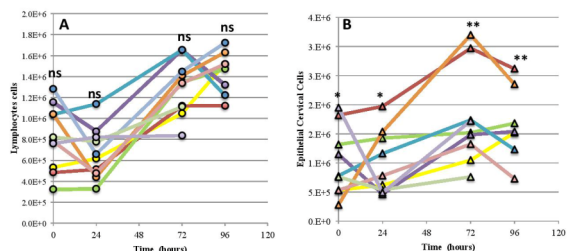
Figure 2. Induction of MN in ten samples healthy human lymphocytes (A) and exfoliated epithelial cervical cells (B) detected by image analysis after treatment with Con-A (5 ug/mL) y Cyt-B (6 ug/mL). The cells were recovered for 24-, 72- and 96-h. A human Lymphocytes with P-value, ns = not significant and Epithelial cells with P-value * < 0.001, P-value **< 0.01. Only ten samples healthy human was showed, other 30 data no show, but have the same behavior.


Figure 3. Induction of MN in ten samples of preneoplastic lesion. We showed human lymphocytes and exfoliated epithelial cervical cell detected by image analysis after treatment with ConA y Cyt-B. The cells were recovered for 24-, 72- and 96-h. A human Lymphocytes with P-value, ns = not significant and Epithelial cells with P-value * < 0.001, P-value **< 0.01. Only ten samples healthy human was showed, other 30 data no show, but have the same behavior.

FIGURE. 4. Induction of MN in human lymphocytes and epithelial cervical cells followed by cell harvest on day 5 of cell culture, at time

zero. P-value***<0.0004, A-B; P-value****<0.0001, C-D; P-value****<0.0001, E-F; P-value, ns = not significant, 0.3678,G-H. A and B healthy groups. C and D, CIN I groups. E and F, CIN II groups. G and H, CIN III groups.
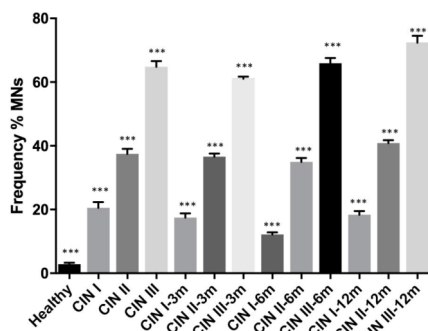
FIGURE. 5. Induction of MN in human lymphocytes followed by cell harvest on day 5 of cell culture, at 0,3,6 and 12 months. P-value***<0.001 vs Control (Healthy).

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