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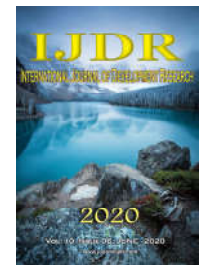
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RESEARCH ARTICLE

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EPIGENETIC FACTORS IN THE EVALUATION OF MICRONUCLEUS FREQUENCY IN BASAL CELLS OF THE UTERINE MUCOSA

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ABSTRACT

Cervical cancer is a tumor which begins in the basal cells of the epithelium lining the uterine cervix, being responsible for approximately 500 thousand new cases in the world each year, becoming also responsible for 230 thousand deaths among women. The research aims to investigate which epigenetic factors interfere in the assessment of the frequency of micronuclei in basal cells of the uterine mucosa. This is an experimental study carried out after approval by the ethics and research committee with the number: 3,679,998. 49 cervical samples were collected from patients who underwent routine gynecological examination in a Basic Health Unit (BHU) in the city of Teresina-PI. A coleta de dados foi efetuada através de questionário e da realização do exame citológico. Cytological analysis was performed using optical microscopy (200X) and/in a blind test in relation to the data obtained in the questionnaire. Shapiro-Wilk chi-square test and Mann Whitney U test were used. The results were distributed in tables, showing the characterization of epigenetic factors, the association of the presence of Micronuclei with sociodemographic and clinical characteristics. The Normality Test was used for the Micronucleus numbers in women and the characterization of epigenetic factors. According to the tests used, there was relevant statistical evidence that the use of X-rays significantly increased the frequency of micronuclei in relation to the other factors studied. This study achieved its listed objectives; it was observed that it is necessary to alert about the risks of frequent X-ray, because these may be associated with the appearance of diseases. The performance of the study is important, since there are few studies that explore the theme in the cervical mucosa.

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INTRODUCTION

Cancer is a serious public health problem, which compromises the well-being of the population, changing the quality of life. Cancer begins with a genetic alteration in the DNA of a cell,

which happens in the proto-oncogenes, which when attacked will turn into oncogenes, modifying healthy cells in cells with cancer (INCA, 2019). According to Leitão *et al.*, (2008), cervical cancer (CC) is a tumor beginning in the basal cells of the epithelium, that lines the uterine cervix, standing out due to

its high incidence and the way it affects the women, becoming the predominant reason for death among them in underdeveloped countries (MENDES *et al.*, 2011). CC is responsible for around 500 thousand new cases in the world each year, being responsible for 230 thousand deaths among women (SOARES *et al.*, 2011). At the national level, this disease occupies the 3rd place in morbidity, where Breast and Colorectal Cancer occupy the 1st and 2nd place respectively, and occupies the 4th most incessant cause of death among women throughout the Brazilian territory. For the biennium (2018-2019), it is estimated that approximately 16,370 new cases will still occur in Brazil (INCA, 2019). Human Papilloma Virus (HPV) infection is considered one of the most common Sexually Transmitted Infections (STI) in the world. Its prevalence in the world population can vary from 3.7% to 57.7%, depending on the region analyzed, being more common in young patients under 25 years of age. Approximately 40% of sexually active women are infected with HPV (RODRIGUES *et al.*, 2014). HPV is a type of virus that affects the skin and mucous membranes of the individual, having direct action on the basal cells of the multi-stratified epithelium, interfering with cellular activity, promoting numerical and structural changes in chromosomes (MENDES *et al.*, 2011). There are several forms of HPV and vary in their tissue tropism, where about 40 types infect the genital tract, and in exposure to genotoxic agents; others are linked to the CC (BRINGHENTI *et al.*, 2010).

Nunes *et al.*, (2013) states that the HPV virus is divided into high and low grade subgroups, according to its oncogenic potential. The low-grade types are types 6, 11, 42, 43 and 44 and are restricted only to dermatological changes that can cause warts, while the high-grade ones 16, 18, 31, 33, 35, 39, 45, 46, 51, 52, 56, 58, 59 and 68 cause precursor lesions of cancer. It is important to emphasize that especially HPV-16 and HPV-18 are responsible for about 70% of cervical cancers, and can be enhanced with indefinite risk factors such as smoking, early onset of sexual activity, multiple partners, alcoholism, immunosuppression, increased life expectancy, thus explaining the meaning of epigenetic factors, which constitutes the combination of epidemiological and genetic factors (INCA, 2019). Due to these chromosomal changes in the basal cells affected by the HPV virus in the cervix, the formation of micronuclei (MN), structures present in the cell cytoplasm, can occur due to the loss of whole or fragmented chromosomes. For Teodemiro (2016), the presence of MN in the multiplying cells is a considerable marker of spontaneous or induced chromosomal anomaly. These changes are usually caused by exposure to various risk factors, hence the importance of conducting the study, to protect against factors that can lead to the appearance of diseases.

METHODOLOGY

Type of Study: It is an experimental study, which is when an object of study is determined, variables that would be able to influence it are selected, and the ways of controlling and observing the effects that the variable produces on the object are defined (GIL, 2008).

Characterization of the field of study: The study was carried out at the Basic Health Unit (BHU) Dr. Carlos Alberto Cordeiro of the Family Health Strategy (ESF) of the Southeast Zone, in Dirceu Arcoverde district, City of Teresina-PI, in November (2019).

This unit offers specialized assistance in order to facilitate the access of the population and to decongest the flow of patients in large hospitals. It is possible to make appointments for General Practice, Pediatrics and Gynecology. In addition, it is prepared to provide care related to Dentistry and Nursing. In parallel to disease prevention, it acts on other primary fronts to protect the health of citizens, such as providing accurate diagnosis and offering appropriate treatment and rehabilitation to patients.

Samples: 49 cervical samples were collected, obtained from patients who underwent routine gynecological examination at the referred BHU. The collection was performed after requesting the Medical and/or Nursing consultation. Patients were asked at the unit about their participation and were asked to sign the Free and Informed Consent Form (FICF). They accepted and signed, a time and place reserved for patients to answer a questionnaire (Appendix A) was set up in the unit itself, and then the collection was made in their own cytology collection room at BHU. The material was collected using an Ayre spatula with an endocervical brush. A part of this collected material was placed on a slide suitable for oncotic cytology; the other part was stored in collection tubes, inside polystyrene with icex, for making slides that were analyzed in relation to the MN in the microbiology laboratory of the Santo Agostinho University Center - UNIFSA.

Inclusion and exclusion criteria: The women who participated in this study meet the inclusion criteria such as: age between 18 to 64 years of age, who are registered in the Unified Health System (SUS), with active sexual life, with clinical suspicion of HPV and/or with cervical injury, as well as patients without any gynecological problems. The exclusion criteria will be: women in the menstrual period; with pre-existing malignant uterine neoplasia and infectious diseases; carriers of other STI already confirmed, such as HIV; and patients who are in the pregnancy period.

Data Collection Procedure: All study participants underwent a routine gynecological examination, which was performed by the researcher after completing a questionnaire applied by the participating researchers, containing information such as age, weight, height, date of last menstruation, birthplace, race, marital status, parity, occupation, sleep, physical activity, frequency of physical exercise, smoking or not, use of medication, alcoholic beverage, health status, family illness, cancer in the family, hereditary illness, sexarche, abortion occurrence, and X-rays in the last year. Some of these factors were statistically correlated with the presence of epigenetic results on the prepared slides. At BHU there is a standard model recommended by the Ministry of Health on data collection and preparation of slides for analysis. The results of the study will be presented as an article to UNIFSA, and an informative banner will be presented to BHU participating in the study, thus participants can become aware of it.

Legal And Ethical Aspects: This study followed Biosafety standards and was carried out with the authorization of the Ethics and Research Committee of the Municipal Health Foundation of the city of Teresina and the Research Ethics Committee (CEP) of the Santo Agostinho University Center with the number 3.679.998, complied with CNS/MS Resolution 466/2012. Sample collection was performed at the BHU of ESF, after specifying information about the research

to each patient, who in turn expressed their spontaneous desire to participate, by signing the informed consent form.

Data Analysis: From the collection of material from the uterine cervix (using a cytobrush), a smear on a glass slide was made by the researchers, in which two drops of saline solution (0.9% NaCl) were added. The fixation of the material was done with a solution of methanol/acetic acid (3: 1) and after 24 hours, the preparations were stained by the method of Feulgen & Rossenbeck (1924) and counter-stained with 1% Fast green, in absolute alcohol for 1 minute. The slides with the collected material were dried at room temperature, and were fixed with Sacomã fixative, in a 3:1 methanol/acetic acid solution. After 24 h, hydrolysis in hydrochloric acid solution (5N) was performed for 15 min, followed by washing in distilled water three times. The staining was done using the Schiff's reagent and the counter staining, with Fast Green 0.2% 1%. The coverslips were assembled with Entelan®. The cytological analysis took place at the UNIFSA General Biology Laboratory under optical microscopy (200X) and in a blind test in relation to the data obtained in the questionnaire. 2,000 cells were analyzed per patient. The micronucleus identification criteria adopted were those described by Tolbert, Shy and Allen (1991; 1992), who consider micronuclei rounded structures and distinctly separated from the nucleus, with well-defined limits, with chromatinic structure and similar coloring, in addition to being seen in same plan.

The collected data were transferred from the data capture form to the Microsoft Excel spreadsheet and then exported to the IBM SPSS Statistics 21.0 program. The statistical analysis was performed by reading the absolute (N) and relative (%) frequencies when it comes to the categorical variable. In the case of the quantitative variable, the measures of position, mean, dispersion, and standard deviation were calculated. And the Chi-square association test was also used, at the 5% significance level, which according to Beiguelman (1996) is a hypothesis test that aims to find a dispersion value for two nominal categorical variables and to evaluate the existing association between qualitative variables and the normality test, which are used to determine whether a set of data for a given random variable is well modeled by a normal distribution or not, or to calculate the probability that the underlying random variable is normally distributed (NASCIMENTO et al., 2017), in addition to the Mann Whitney U Test.

RESULTS

Initially, Table 1 shows the socioeconomic characterization of the samples in women who underwent the gynecological examination at the BHU. In Table 1, the patients were characterized according to the presence of MN, age group, race, civil status, number of children, physical activity, and education. Regarding the number of MN, the study revealed that 44.9% of patients had MN. As for the age group, it was observed that 51.0% are young adult portion (20 - 40 years). The brown race stood out, with 67.3%. Regarding civil status, the vast majority are married and single, making up 98% of the sample. On average the participants have 2 children and 51% do not practice physical activity. With regard to the education of the participants 57.1% have finished high school or have not completed it. Table 2 shows the clinical characterization of patients who underwent routine cytopathological examination

at the referred BHU. Regarding cancer, 63.4% of the participants do not have this disease (8 women did not know how to answer it when asked about this question) and 83.7% do not make continuous use of hormones. Regarding the consumption of alcoholic beverages, 42.9% do not use it. 81.6% deny the use of tobacco and 61.2% do not have comorbidities. We can also see that 75.5% of women had an abortion. About the use of x-rays, significant differences were found for genetic abnormalities of patients who underwent this examination in the last year, with 81.6% of the sample. Table 3 describes the relationship between the sociodemographic and clinical epigenetic factors searched in the sample with the frequency of MN in basal cells of the uterine mucosa. This relation is observed when the *p-value* is less than 5%, these data showed a non-significant trend between the low frequency of MN and the absence of risk factors such as age, race, marital status, education, physical activity, cancer, hormone use, drinking, smoking, illness and abortion. The use of X-rays significantly increased the frequency of MN in relation to groups that did not have this factor. Therefore, based on the Chi-square test of association, there is a 5% significance level, statistical evidence shows a connection between the presence of MN and the use of X-rays. The normality test was performed according to the level of significance at 5% for the number of MN in women who underwent routine gynecological examination at the BHU. According to the Normality Test, based on the analysis of the *p-value*, there is statistical evidence that the distribution is not within the normal range as there were women who had MN and women who did not.

It can be observed that according to Mann Whitney U Test for comparison of independent groups, only the number of children was statistically significant in the variable Number of children, considering the age and Number of abortions, they did not present statistical relations significant. Table 7 shows the comparison between the number of MN and the epigenetic factors in women who were examined. Factors such as cancer, smoking, use of hormones and alcoholic beverages did not show a statistically significant relation in the number of MN, different from the patients who underwent the X-ray exam in the last year, which presented statistical relevance according to the proposed questionnaire (Appendix A).

DISCUSSION

The purpose of the MN technique has become a predominant biomarker for human presentation to mutagenic and clastogenic agents and has been shown to be sensitive to low levels of exposure, including effects of exposure to industrial chemicals and air pollution (BENITES *et al.*, 2006). The application of the MN technique to cervical cells in cytological smears, with cells in normal conditions, in the process of inflammation, and dysplasia, was made by Gattás *et al.*, (2001), where they observed a multiplication in the frequency of MN. A similar effect occurred in this study, where an increase in the frequency of MN was observed in the patients studied. Several factors can affect the response of cells, including age, sex, genetic composition and exogenous factors, which are directly related to the lifestyle of a person, as well as smoking, drinking alcohol, using oral contraceptives, among others (CAMPOS *et al.*, 2008; VILANOVA *et al.*, 2012). Paulino (2012) when correlating in his study the age range of patients with the appearance of MN found that there is no

Table 1: Socioeconomic characterization of cervical samples in women who underwent the gynecological examination at the BHU. (n= 49). Teresina- PI, 2019

Variables	N (%)	Mean	Min	Max	SD
Presents MN					
Yes	22 (44.9)				
No	27 (55.1)				
Age Group					
Young (<20 years old)	8 (16.3)				
Young Adult (20-40 years old)	25 (51.0)				
Elderly Adult (40-60 years old)	16 (32.7)				
Race					
White	7 (14.3)				
Black	9 (18.4)				
Brown	33 (67.3)				
Civil Status					
Married	22 (44.9)				
Single	26 (53.1)				
Widow	1 (2.0)				
Number of children		2	0	6	1
Physical activity					
Yes	24 (49.0)				
No	25 (51.0)				
Education degree					
Elementary School	10 (20.4)				
High School	28 (57.1)				
Higher Education	11 (22.4)				

Source: Direct search (2019).

Table 2. Clinical characterization of cervical samples in women who underwent gynecological examination at the BHU (n= 49). Teresina- PI, 2019

Variables	N (%)
X-rays	
Yes	9 (18.4)
No	40 (81.6)
Smoke	
Yes	9 (18.4)
No	40 (81.6)
Drinking	
No	28 (57.1)
Yes	21 (42.9)
Oral Contraceptives	
Yes	8 (16.3)
No	41 (83.7)
Diseases	
Yes	19 (38.8)
No	30 (61.2)
Cancer	
Yes	15 (36.6)
No	26 (63.4)
Abortion	
Yes	12 (24.5)
No	37 (75.5)

Source: Direct search (2019).

difference, both for patients younger than or equal to 25 years old, and for those older than this, which confirms the result of this study, where there was no alteration. The study records of the connection between the occurrence of MN and physical activity are scarce. The study conducted by Queiros (2017) found that physical exercise causes severe changes in the chromosomal level in blood lymphocytes, and pointed out statistical relevances that show the increase in MN in people who practice physical activity, contradicting the results of the present study that had no evidence relating this factor and the increase in MN. Andrade *et al.* (2005) shows in their study that after the removal of the carcinogens, there was a rapid decrease in micronuclei. This evidence is confirmed by the reduction of these chromosomal defects observed after the treatment of malignant neoplasms with radiotherapy, when compared to the count of those that were not irradiated, that is, the cancer according to him is related.

However, this study shows that there was no link between the appearance of MN and cancer. Regarding the consumption of alcoholic beverages, Batista and Campos (2015) in their study did not find any significant results for the increase in MN, which compared to this study there was no statistically significant evidence.

Stich *et al.* (1982), researching the relevance of the MN test in analyzing the exposure of people to chemical genotoxic agents such as tobacco in individual proportions, expressed that the frequency of MN depicts a higher value in the associated form. After one year, the study by Stich *et al.* (1982) reported that through the MN test in smoking and non-smoking patients, they saw that the frequency of MN in smokers is highlighted, compared to individuals who do not smoke. Also according to Stich *et al.*, (1982), they also performed the analysis of MN in scrapes of the mucosa of smokers.

Table 3: Association of the presence of MN and the sociodemographic and clinical characteristics of cervical samples in women, who underwent routine gynecological examination at the UBS). (n= 49). Teresina- PI, 2019.

Variables	N	Presents MN		p-value
		Yes %	No %	
Age Group				0.131
Young (<20 years old)	5	10.20%	3 6.10%	
Young Adult (20 -40 years old)	13	26.50%	12 24.50%	
Elderly Adult (40 -60 years old)	4	8.20%	12 24.50%	
Race				0.076
White	4	8.20%	3 6.10%	
Black	1	2.00%	8 16.30%	
Brown	17	34.70%	16 32.70%	
Civil Status				0.388
Married	11	22.40%	11 22.40%	
Single	10	20.40%	16 32.70%	
Widow	1	2.00%	0 0.00%	
Physical activity				0.897
Yes	11	22.40%	13 26.50%	
No	11	22.40%	14 28.60%	
Education degree				0.512
Elementary School (Incomplete/Complete)	3	6.10%	7 14.30%	
High School (Incomplete/Complete)	13	26.50%	15 30.60%	
Higher Education (Incomplete/Complete)	6	12.20%	5 10.20%	
X-rays				0.024
Yes	1	2.00%	8 16.30%	
No	21	42.90%	19 38.80%	
Smoke				0.44
Yes	3	6.10%	6 12.20%	
No	19	38.80%	21 42.90%	
Drinking				0.74
No	12	24.50%	16 32.70%	
Yes	10	20.40%	11 22.40%	
Oral Contraceptives				0.751
Yes	4	8.20%	4 8.20%	
No	18	36.70%	23 46.90%	
Diseases				0.367
Yes	7	14.30%	12 24.50%	
No	15	30.60%	15 30.60%	
Cancer				0.786
Yes	7	17.10%	8 19.50%	
Table 3, continuation				
No	11	26.80%	15 36.60%	
Abortion				0.282
Yes	7	14.30%	5 10.20%	
No	15	30.60%	22 44.90%	

Source: Direct search (2019). * Chi-square association test, at a 5% significance level.

Table 4: Normality test for MN numbers in women who underwent routine gynecological examination at the BHU. (n= 49). Teresina- PI, 2019

	Statistic	df	p-valor
Number of MN	0,533	49	<0,001

Source: Direct search (2019). * Normality test, at a significance level of 5%.

Table 5: Comparison between the presence of MN and age, number of children and number of abortions in women who underwent routine gynecological examination at the BHU (n= 49). Teresina- PI, 2019

Variables	Presents MN		p-value
	Yes	No	
Age	Mean (SD) 30 (12)	Median 27	0.082
Number of children	1 (1)	1	0.045
Table 5, continuation			
Number of abortions	0.36 (0.58)	0.00	0.356

Source: Direct search (2019). * Mann Whitney U test, at 5% significance level.

Table 6- Comparison between the number of MN and the Epigenetic Factors in women who underwent routine gynecological examination at the BHU. (n= 49). Teresina- PI, 2019.

Variables	Mean (SD)	Median	p-value
X-rays			0.044
Yes	0.22 (0.667)	0	
No	4.28 (7.72)	1	
Smoke			0.379
Yes	0.66 (1.118)	0	
No	4.175 (7.769)	0	
Drinking			0.782
No	4.52 (8.495)	0	
Yes	2.79 (6.00)	0	
Oral Contraceptives			0.905
Yes	3.5 (7.95)	1	
No	3.53 (7.09)	0	
Cancer			0.841
Yes	4.46 (8.34)	0	
No	2.46 (5.874)	0	

Source: Direct search (2019). * Mann Whitney U test, at 5% significance level.

This and other researches show that the consumption of tobacco in its various ways causes an increase in MN. However, Karahalil *et al.*, (1999) affirm that this may be the result of methodological questions, such as the quantity of cigarettes, and difference in the response capacity. Studies report that the consumption of more than 20 cigarettes/day may cause irreversible damage to a greater number of cells (SARTO *et al.*, 1987). Thus justifying the result of this study, where it did not show results of this research, in relation to the increase in MN and the smoking habit.

Carrard *et al.*, (2007) report that a very large number of diseases have been investigated as to their relation with the presence of micronuclei, some of which have already been confirmed and others need more research. Among the diseases, we have CC, AIDS, among others. Our study showed the opposite, with no relation between the women studied and the increased frequency of MN. Regarding the use of oral contraceptives, the study by Aieres (2008) confirms the result of this research, as the author reports in his study that there were no differences in the occurrence of these structures due to sexual variables (age at first intercourse and number of sexual partners), use of hormonal contraceptives, multiparity and HPV infection. X-rays are a potent mutagenic agent, capable of inducing genetic mutations and chromosomal aberrations, acting directly on the DNA molecule or indirectly through the formation of reactive components that interact with this molecule (CERQUEIRA *et al.*, 2008). Considering that an individual may be subjected to repeated radiographs throughout life, Antonio *et al.*, (2017) found that the effect of successive exposures to X-rays and repetitions of this should be considered, as there may be an increase in the frequency of nuclear changes after these events. Thus, the cumulative effect of small doses on sensitive tissues could trigger cytotoxic effects, resulting in chronic cell aggression, compensatory cell proliferation, development of tumors and carcinogenesis. Corroborating the results of this study, Soma *et al.*, (2012) observed in their research that the MN frequencies were high, in relation to the X-ray exam, determining cells with two to four MN in patients. Increasing evidence suggests that radiographs used widely as a diagnostic tool can induce cytotoxic effects and cause DNA damage. In addition, the nucleus (including its genetic material) is more radiosensitive than the cell cytoplasmic structures (SHEIKH *et al.* 2012). Factors such as race, civil status, number of children and education have studies related to the oral mucosa. As for the cervical mucosa, there was not much scientific evidence to support the study.

Conclusion

The present study achieved its listed objectives, as it was possible to investigate the epigenetic factors that interfere in the assessment of the frequency of MN, in addition to characterizing the study participants and describing these factors. The result related the exposure to X-ray to the increase in MN in the research participants, who underwent this exam in the last year, confirmed through the Chi-square test of association at a significance level of 5%. As the female population is the most frequent in the search for disease prevention and treatment, it is necessary to alert them to the risks that the X-ray brings, because the increase in MN, may be associated with other pathologies, which if not diagnosed early can cause irreversible damage to the health of these women and lead them to death. It is important that women

know all the factors that can cause a genetic change in their body, considering that these changes are associated with the emergence of serious pathologies capable of compromising their quality of life. Therefore, the realization of this study is pertinent because it allows the follow-up of new researches that lead to complement the lines expressed from this, and serve as support for health professionals and students, exacerbating their knowledge on the exposed theme, since there are few exploring the theme of the cervical mucosa.

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