AOGS
ACTA Obstetricia et Gynecologica Scandinavica

MAIN RESEARCH ARTICLE

Cervical precancerous lesions – chromosomal instability in peripheral blood lymphocytes in relation to lesion stage, age and smoking habits

OLIVERA MILOŠEVIĆ-DJORDJEVIĆ[1,2], IVANA STOŠIĆ[1], DARKO GRUJIČIĆ[1], DRAGIĆ BANKOVIĆ[1] & SLOBODAN ARSENIJEVIĆ[2]

[1]*Faculty of Science and* [2]*Faculty of Medicine, University of Kragujevac, Kragujevac, Serbia*

Key words
cervical precancerous lesions, peripheral blood lymphocytes, lesion stage, micronuclei, chromosomal instability

Correspondence
Olivera Milošević-Djordjević, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, PO Box 60, 34 000 Kragujevac, Serbia.
E-mail: olivera@kg.ac.rs

Conflict of interest
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Received: 9 December 2010
Accepted: 21 June 2011

DOI: 10.1111/j.1600-0412.2011.01230.x

Abstract

Objective. To evaluate chromosomal damage in peripheral blood lymphocytes (PBL) of patients newly diagnosed with cervical precancerous lesions with respect to age, smoking habits, miscarriages, abortions and lesion stage. *Design*. Clinical study. *Setting*. Clinic of Gynecology and Obstetrics in Kragujevac, Serbia, during 2009-2010. *Population*. The analyzed samples included 41 untreated patients aged 24–65years with a diagnosed low-grade squamous intraepithelial lesion (LSIL; 19 patients) or a high-grade squamous intraepithelial lesion (HSIL; 22 patients). Control samples were obtained from 40 healthy women aged 24–53 years. *Methods*. The frequency of micronuclei (MN) was estimated in circulating lymphocytes by using the cytokinesis-block micronucleus assay. *Main Outcome Measure*. The frequency of MN in PBL. *Results*. The mean MN frequencies of both LSIL and HSIL patients were significantly higher than the MN frequency of healthy control women. There was no significant difference in MN frequency between LSIL and HSIL patients, between smokers and nonsmokers in both patient and control samples, or between miscarriage groups and abortion groups of patients. Considering confounder factors, age and health status influenced MN frequency. *Conclusions*. The results suggest that MN frequency in PBL of patients with cervical precancerous lesions corresponds to an increase of chromosomal damage, irrespective of smoking habits, miscarriages, abortions and lesion stages.

Abbreviations: BN, binucleated; HPV, human papilloma virus; HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; MN, micronuclei; PBL, peripheral blood lymphocytes

Introduction

Cervical cancer is the second most common female malignancy. Cervical cancer develops through a multistep process that includes either three (cervical intraepithelial neoplasia or CIN I–III) or, according to the Bethesda system, two lesion stages [low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL)]. When the lesions do not progress, they either regress or persist at the same grade. Low-grade squamous intraepithelial lesions are likely to regress, while HSIL are more likely to progress towards cancer (1).

Chromosomal instability is a main characteristic of cancer (2–5). Considering that cancerogenesis is a complex stepwise process and that the accumulation of genetic alterations allows growth of neoplastic cells, chromosomal instability is a possible event in the precancerous stages. This assumption has been confirmed through numerous studies showing that chromosomal instability occurs in precancerous lesions. Desai et al. (6) demonstrated that oral precancerous lesions are associated with an increased number of micronuclei (MN) both in circulating lymphocytes and in oral exfoliated cells. Likewise, Saran et al. (7) observed a significant stepwise increase of micronucleated cells and micronuclei in buccal epithelial cells from control to oral precancer patients. Increased baseline DNA damage was shown by applying the Comet assay in buccal cells and lymphocytes (7).

Singh et al. (8) observed chromosomal abnormalities (aneuploidy) in cervical cells of women with LSIL and HSIL. The chromosomal instability was reported as an increased level of cytogenetic biomarkers. An increased frequency of spontaneous chromosome aberrations was observed among patients with cervical precancerous lesions (9), as well as increased sister chromatid exchange frequency (10).

The presence of MN reveals chromosomal damage in cells, and there is a positive correlation between these two endpoints. The micronucleus has the appearance of a small nucleus in the cell cytoplasm, originating from chromosome fragments or whole chromosomes that are lagging behind during nuclear division. Generally, micronuclei are induced by oxidative stress or by exposure to clastogenic or aneugenic agents (11). The MN assay is effective in determining the genotoxic impact of environmental and lifestyle factors (12–14), as well for predicting individual risk assessment (11,15).

As the MN assay is a useful method for assessing chromosomal damage and because MN in lymphocytes can reflect cancerogenesis in target tissues, the objective of this study was to evaluate MN frequency in peripheral blood lymphocytes (PBL) in untreated women with diagnosed precancerous lesions (LSIL and HSIL) and in healthy control women in relation to lesion stage, age, smoking habits, miscarriages and abortions.

Material and methods

This study was approved by the Ethics Committee of the Clinic of Kragujevac (number 01/2577). The analyzed samples included 41 newly diagnosed patients (19 with diagnosed LSIL and 22 with diagnosed HSIL), aged 24–65years, observed at the Clinic of Gynecology and Obstetrics in Kragujevac, Serbia during 2009–2010. No patient had been treated or been occupationally exposed to known mutagenic agents. All patients had a gynecological examination, including a Papanicolau smear. Fourteen patients had a history of miscarriage, and 26 a history of abortions. Twenty were smokers. Control samples were from 40 healthy female donors aged 24–53years, without any recent exposure to known mutagenic agents, and 17 were smokers.

Micronucleus test

The cytokinesis-block method described by Fenech (16) was used. Peripheral blood samples from patients and healthy controls were collected in a heparinised sterile syringe. Whole heparinised blood (0.5ml) was added to 5ml complete medium for the lymphocytes cultivation PBMax Karyotyping (Invitrogen, California, USA). All cultures were set up in duplicate and were incubated at 37°C for 72hours. Cytochalasin B (Sigma, St. Louis, MO, USA) at a final concentration of 4μg/ml was added 44hours after the beginning the cultivation. After incubation for another 28hours, cells were collected by centrifugation and treated with cold (4°C) hypotonic solution (0.56% KCl). Cell suspensions were fixed in freshly prepared acetic acid-methanol (1:3) three times and were dropped onto specially prepared clean and cold slides. Lamp-dried slides were stained in 2% Giemsa solution (Alfapanon, Novi Sad, Serbia) for 12minutes.

Micronucleus frequencies were determined by analyzing 1000 binucleated (BN) cells per individual, according to criteria described by Fenech (17). Slides were examined under a light microscope (Nikon E50i) at ×400 magnification.

Statistical analysis

The results are shown as mean±standard deviation (SD). The mean MN frequencies of LSIL, HSIL and healthy control women were analyzed by ANOVA and Bonferroni's test for multiple comparisons. Student's *t*-test was used for comparing the MN frequencies between smokers and nonsmokers, age groups in LSIL patients and control women and for miscarriage and abortion groups in patient sample. The mean MN frequencies of LSIL patients aged ≤40years and aged-matched HSIL patients and MN frequencies in age groups of HSIL patients were compared by using the Mann–Whitney *U*-test. The influence of age, smoking status and health status on MN frequency in analyzed sample was determined by the application of multiple linear regression analyses. Levels of significance were $p<0.05$, $p<0.01$ and $p<0.001$.

Results

Results of the MN assay in PBL of patients with newly diagnosed precancerous cervical lesions and healthy control women are shown in Tables 1–4.

Table 1 presents general and lifestyle characteristics of patients and healthy control women. Table 2 shows the mean MN frequencies in both patient and control samples. The mean baseline MN frequency was significantly higher ($p<0.0005$) in both LSIL and HSIL patient samples (14.26±4.49 and 15.95±4.27 per 1000 BN cells, respectively) when compared with the baseline MN frequency in healthy control women (7.15±3.17 per 1000 BN cells). The mean MN frequencies among LSIL and HSIL patients were not significantly different ($p=0.484$). The mean frequencies of MN did not significantly differ between smokers and nonsmokers in both patient and control samples ($p=0.150$ and $p=0.965$, respectively). Likewise, there were no significant differences in MN frequencies between miscarriage patient groups ($p=0.906$), as well as between abortion patient groups ($p=0.488$). In LSIL, the patient's age had a significant effect on the MN frequency ($p=0.008$); however, in HSIL patients and in control samples, age had no significant effect ($p=0.135$ and $p=0.438$, respectively). The LSIL patients aged ≤40years

Table 1. General characteristics of the analyzed samples.

Analyzed samples	Number
Control women	
Total	40
Age (mean±SD, range)	38.87±8.05 (24–53)
Smoking habit	
Smokers	17
Nonsmokers	23
Patients	
Total	41
LSIL	19
HSIL	22
Total age (mean±SD, range)	41.34±11.72 (24–65)
LSIL	42.58±12.19 (24–64)
HSIL	40.27±11.48 (24–65)
Smoking habit	
Smokers	20
Nonsmokers	21
Miscarriages (*n*, range)	
No	27
Yes	14 (1–3)
Abortions (*n*, range)	
No	15
Yes	26 (1–4)

Abbreviations: HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions.

had significantly lower MN frequency than HSIL patients aged ≤40years (p=0.044).

Table 3 shows the results of MN distribution in analyzed BN cells. Cells with one MN were mostly present in both patients and control samples. Of the 41 000 analyzed BN cells among patients, 532 (1.30%) had one MN, compared with 276 (0.69%) in the 40 000 analyzed BN cells of the control samples. Cells with two MN were less common (0.08% in HSIL patients, 0.05% in LSIL patients and 0.008% in control women). Cells with three, four or five MN were predominant in patient samples.

Multiple linear regression analyses showed that among confounder factors, age and health status had an effect on the MN frequency in the analyzed study sample (p=0.005 and p<0.0005, respectively). The effect of smoking on the MN frequency was not significant (p=0.469;Table 4).

Discussion

Cervical squamous intraepithelial lesions (LSIL and HSIL) are a precancerous stage of cervical cancer. Chromosomal instability is a common event in cervical cancer patients (18–20), and it is thought that chromosomal instability will also be elevated in precancerous stages. These statements were confirmed by using a variety of tests, such as chromosome aberration (9), sister chromatid exchange (10) and the MN test (21).

Table 2. Frequency of micronuclei in peripheral blood lymphocytes of patients with precancerous lesions and healthy control women.

	Mean±SD (range)	
	Patients	Control women
Total number	41	40
MN		
State of lesion		
LSIL	14.26 ± 4.49(6–23)[a,b]	
HSIL	15.95 ± 4.27(11–26)[c]	7.15 ± 3.17(1–14)
Smoking habits		
Smokers	14.15 ± 4.29(6–26)[d]	7.18 ± 3.34(1–14)[e]
Nonsmokers	16.14 ± 4.38(10–24)	7.13 ± 3.11(1–13)
Miscarriage		
No	15.11 ± 4.18(10–26)[f]	
Yes	15.29 ± 4.97(6–24)	
Abortion		
No	14.53 ± 3.48(10–21)[g]	
Yes	15.54 ± 4.88(6–26)	
Age groups (years)		
LSIL	11.56 ± 3.36(24–40)[h,i]	
	16.70 ± 4.06(41–64)	
		6.83 ± 3.55(24–38)[k]
		7.59 ± 2.60(39–53)
HSIL	15.25 ± 4.99(24–40)[j]	
	16.80 ± 3.26(41–65)	

Abbreviations: HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; MN, micronuclei.

[a] No significant difference in MN frequencies between LSIL and HSIL patients, p=0.484 (Bonferroni's test).

[b] Statistically significant difference in MN frequencies between LSIL and control women, p<0.0005 (Bonferroni's test).

[c] Statistically significant difference in MN frequencies between HSIL and control women, p<0.0005 (Bonferroni's test).

[d,e] No significant difference in MN frequencies between smokers and nonsmokers in patients and healthy control women, p=0.150 and p=0.965, respectively (Student's t-test).

[f] No significant difference in MN frequencies between miscarriage groups of patients, p=0.906 (Student's t-test).

[g] No significant difference in MN frequencies between abortion groups of patients, p=0.488 (Student's t-test).

[h] Statistically significant difference in MN frequencies between age groups of LSIL patients, p=0.008 (Student's t-test).

[i] Statistically significant difference in MN frequencies between LSIL and HSIL patients aged ≤40, p=0.044 (Mann–Whitney U-test).

[j] No significant difference in MN frequencies between age groups of HSIL patients, p=0.135 (Mann–Whitney U-test).

[k] No significant difference in MN frequencies between age groups of healthy control women, p=0.438 (Student's t-test).

Recently published studies confirmed that chromosomal instability in cancer cells and cancer-prone cells can be evaluated by the presence of MN in lymphocytes (5,11,22). Lymphocytes are long-lived cells and, while circulating through organs, they are exposed to different tissue products that may affect DNA damage. Therefore, MN in lymphocytes can be used as a relevant biomarker for health risk.

Table 3. Distribution of micronuclei in peripheral blood lymphocytes of analyzed cervical precancerous patients and control women.

Sample	Analyzed BN cells	BN cells with MN (%)	Distribution of MN (%)				
			1	2	3	4	5
Patients	41 000	570 (1.39)	532 (1.30)	27 (0.07)	9 (0.02)	1 (0.002)	1 (0.002)
LSIL	19 000	249 (1.31)	235 (1.24)	9 (0.05)	3 (0.02)	1 (0.005)	1 (0.005)
HSIL	22 000	321 (1.46)	297 (1.35)	18 (0.08)	6 (0.03)	0 (0)	0 (0)
Control women	40 000	280 (0.7)	276 (0.69)	3 (0.008)	0 (0)	1 (0.003)	0 (0)

Abbreviations: BN, binucleated; HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; MN, micronuclei.

Table 4. Results of multiple regression analyses for micronucleus frequency, including age, smoking habits and health status.

		Unstandardized coefficients		Standardized Coefficients		
		B	SEM	β	t	p-Value
Model 1	(Constant)	10.023	2.394	–	4.187	0.000
	Age	0.143	0.049	0.261	2.901	0.005
	Smoking habits	0.719	0.988	0.065	0.728	0.469
	Health status	−3.889	0.591	−0.573	−6.579	0.000

In our study, the highest mean MN frequency was found in the lymphocytes of HSIL patients, while the lowest was found in healthy control women. Generally, both LSIL and HSIL patients had an increased MN frequency in PBL when compared with healthy control women, suggesting that chromosomal instability (chromosomal losses or breakages) is a common event in cervical cancerogenesis. Our results are in agreement with a study by Leal-Garza et al. (21), in which LSIL and HSIL patients had significantly different MN frequencies than the control women in both lymphocytes and cervical epithelium cells. Likewise, Guzmán et al. (23) reported an increased frequency of micronucleated cells in exfoliated cervical cells of women with both LSIL and HSIL diagnoses.

Considering lesion stages, LSIL patients had lower mean MN when compared with HSIL patients, but without statistical significance. Similarity in the MN frequency between LSIL and HSIL patients may be correlated with the age of LSIL patients (42.6±12.19 vs. 40.3±11.48years), where age affects the MN frequency.

One possible explanation for increased chromosome damage in cervical precancerous lesions can be infection with high-risk human papilloma viruses (HPV). The vast majority of diagnosed lesions are in women who have an HPV infection (24,25). Alvarez-Rosero et al. (26) observed that there is a correlation between the presence of high-risk HPV infection in cervical cells and the induction of genomic instability in lymphocytes. Likewise, in a study by Cortés-Gutiérrez et al. (27), women with HPV infection had a higher MN frequency in cervical cells. Apart from these reasons, clastogenic products released by tumor cells (28) may be responsible for the increased persistence of micronuclei in lymphocytes.

Our results showed great interindividual variation in lymphocyte MN frequency in both patient and control samples. Personal characteristics, such as those relating to lifestyle, age, individual susceptibility or exposure to different mutagens, might contribute to this variability. Several confounding factors that can contribute to the baseline MN frequency have been elaborated. Results from the HUMAN MicroNucleus project (13) detailing smoking habits indicated that MN frequency significantly increased in PBL of some groups of smokers, but not in all. Likewise, there was no effect of smoking on the lymphocyte MN frequency in cancer patients (29). In our study samples, we found no significant difference in MN frequency between smokers and nonsmokers. Furthermore, in the patient sample the smokers had a lower mean MN frequency when compared with nonsmokers. As a possible reason for this result, Bonassi et al. (13) concluded that tobacco-damaged lymphocytes may not survive the culture period of the assay or may not divide.

The role of age as a potential confounder was also investigated in cytogenetic studies. A number of studies demonstrated a correlation between age and MN frequency (12,30,31), but several studies have not demonstrated an effect of age on MN frequency (32–34). In our study, there was a significant correlation between age and MN frequency.

Comparing the MN of two age groups (≤40 vs. >40years) in both LSIL and HSIL patients, our results indicate significantly greater chromosomal damage in LSIL patients

>40years. Furthermore, our results indicate significantly greater chromosomal damage in PBL of HSIL patients ≤40years old when compared with age-matched LSIL patients.

It is possible that baseline genetic damage can be modulated by different endogenous factors, including genes involved in the metabolism of different genotoxins. It has been revealed that polymorphism in genes, such as *EPHX* (epoxide hydrolase), *GSTT1* (glutathione *S*-transferase T1) and *GSTM1* (glutathione *S*-transferase M1), are of special importance in modulating the frequency of chromosomal damage in populations exposed to genotoxic agents and unexposed individuals (35).

In addition to the increase of MN frequency in PBL of patients, our data demonstrated an increase in the number of BN cells that contain MN, as well as the number of BN cells with more than one MN per BN cell. Considering that the number of MN reflects the level of chromosomal damage, PBL of patients had more chromosomal damage in comparison to healthy control women. The MN frequency in PBL of patients with cervical precancerous lesions is associated with an increase of chromosomal instability, irrespective of smoking habits, miscarriages, abortions and lesion stages. The increase of MN frequency in patients corresponds to an increase in chromosomal damage occurring at the molecular basis of disease. We suggest that MN frequency in PBL can be a relevant biomarker for a predisposition to cervical precancerous lesions.

Funding

The study was supported by the Ministry of Education and Science of the Republic of Serbia (grant no. 41010).

References

1. Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol. 1993;12:186–92.
2. Colleu-Durel S, Guitton N, Nourgaileva K, Leveque J, Danic B, Chenal C. Genomic instability and breast cancer. Oncol Rep. 2001;8:1001–5.
3. Hamurcu Z, Donmez-Altuntas H, Patiroglu T. Basal level micronucleus frequency in stimulated lymphocytes of untreated patients with leukemia. Cancer Genet Cytogenet. 2008;180:140–4.
4. Joseph LJ, Bhartiya US, Raut YS, Kand P, Hawaldar RW, Nair N. Micronuclei frequency in peripheral blood lymphocytes of thyroid cancer patients after radioiodine therapy and its relationship with metastasis. Mutat Res. 2009;675: 35–40.
5. Milošević-Djordjević O, Grujičić D, Vasković Z, Marinković D. High micronucleus frequency in peripheral blood lymphocytes of untreated cancer patients irrespective of gender, smoking and cancer sites. Tohoku J Exp Med. 2010;220:115–20.
6. Desai SS, Ghaisas SD, Jakhi SD, Bhide SV. Cytogenetic damage in exfoliated oral mucosal cells and circulating lymphocytes of patients suffering from precancerous oral lesions. Cancer Lett. 1996;109:9–14.
7. Saran R, Tiwari RK, Reddy PP, Ahuja YR. Risk assessment of oral cancer in patients with pre-cancerous states of the oral cavity using micronucleus test and challenge assay. Oral Oncol. 2008;44:354–60.
8. Singh M, Mehrotra S, Kalra N, Singh U, Shukla Y. Correlation of DNA ploidy with progression of cervical cancer. J Cancer Epidemiol. 2008;2008:298495.
9. Murty VV, Mitra AB, Luthra UK. Spontaneous chromosomal aberrations in patients with precancerous and cancerous lesions of the cervix uteri. Cancer Genet Cytogenet. 1985;1:347–53.
10. Murty VV, Mitra AB, Luthra UK, Singh IP. Sister chromatid exchanges in patients with precancerous and cancerous lesions of cervix uteri. Hum Genet. 1986;72:37–42.
11. Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, et al. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis. 2007;28:625–31.
12. Bolognesi C, Lando C, Forni A, Landini E, Scarpato R, Migliore L, et al. Chromosomal damage and ageing: effect on micronuclei frequency in peripheral blood lymphocytes. Age Ageing. 1999;28:393–7.
13. Bonassi S, Neri M, Lando C, Ceppi M, Lin Y, Chang WP, et al. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human Micro Nucleus project. Mutat Res. 2003;543:155–66.
14. Rekhadevi PV, Mahboob M, Rahman MF, Grover P. Genetic damage in wood dust-exposed workers. Mutagenesis. 2009;24:59–65.
15. Murgia E, Ballardin M, Bonassi S, Rossi AM, Barale R. Validation of micronuclei frequency in peripheral blood lymphocytes as early cancer risk biomarker in a nested case–control study. Mutat Res. 2008;639:27–34.
16. Fenech M. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. Mutat Res. 1993;285:35–44.
17. Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2:1084–104.
18. Lukovic L, Milasin J. Sister chromatid exchange in patients with carcinoma in situ of cervix uteri. Cancer Genet Cytogenet. 1992;59:84–5.
19. Dhillon VS, Kler SR, Dhillon IK. Chromosome instability and sister chromatid exchange (SCE) studies in patients with carcinoma of cervix uteri. Cancer Genet Cytogenet. 1996;86:54–7.
20. Cortes-Gutierrez EI, Cerda-Flores RM, Leal-Garza CH. Sister chromatid exchanges in peripheral lymphocytes from

women with carcinoma of the uterine cervix. Cancer Genet Cytogenet. 2000;122:121–3.
21. Leal-Garza CH, Cerda-Flores RM, Leal-Elizondo E, Cortes-Gutierrez EI. Micronuclei in cervical smears and peripheral blood lymphocytes from women with and without cervical uterine cancer. Mutat Res. 2002;515:57–62.
22. Iarmacovai G, Ceppi M, Botta A, Orsiere T, Bonassi S. Micronuclei frequency in peripheral blood lymphocytes of cancer patients: a meta-analysis. Mutat Res. 2008;659:274–83.
23. Guzmán P, Sotelo-Regil RC, Mohar A, Gonsebatt ME. Positive correlation between the frequency of micronucleated cells and dysplasia in Papanicolaou smears. Environ Mol Mutagen. 2003;41:339–43.
24. Evans MF, Adamson CS, Papillo JL, St John TL, Leiman G, Cooper K. Distribution of Human Papillomavirus types in ThinPrep Papanicolaou tests classified according to the Bethesda 2001 terminology and correlations with patient age and biopsy outcomes. Cancer. 2006;106:1054–64.
25. Zuna RE, Allen RA, Moore WE, Lu Y, Mattu R, Dunn ST. Distribution of HPV genotypes in 282 women with cervical lesions: evidence for three categories of intraepithelial lesions based on morphology and HPV type. Mod Pathol. 2007;20:167–74.
26. Alvarez-Rosero RE, Rodríguez-Argote J, Arboleda-Moreno YY, Muñoz-Benítez SL, Sierra-Torres CH. Chromosome aberrations in peripheral blood lymphocytes of high-risk HPV-infected women with HGSIL. Environ Mol Mutagen. 2008;49:688–94.
27. Cortés-Gutiérrez EI, Dávila-Rodríguez MI, Vargas-Villarreal J, Hernández-Garza F, Cerda-Flores RM. Association between human papilloma virus-type infections with micronuclei frequencies. Prague Med Rep. 2010;111:35–41.
28. Werkmeister J, Zaunders J, McCarthy W, Hersey P. Characterization of an inhibitor of cell division released in tumour cell cultures. Clin Exp Immunol. 1980;41:487–96.
29. Duffaud F, Orsiere T, Villani P, Pelissier AL, Volot F, Favre R, et al. Comparison between micronucleated lymphocyte rates observed in healthy subjects and cancer patients. Mutagenesis. 1997;12:227–31.
30. Bolognesi C, Abbondandolo A, Barale R, Casalone R, Dalpra L, De Ferrari M, et al. Age-related increase of baseline frequencies of sister chromatid exchanges, chromosome aberrations, and micronuclei in human lymphocytes. Cancer Epidemiol Biomarkers Prev. 1997;6:249–56.
31. Fenech M. Important variables that influence base-line micronucleus frequency in cytokinesis-blocked lymphocytes – a biomarker for DNA damage in human populations. Mutat Res. 1998;404:155–65.
32. Trková M, Kapras J, Bobková K, Stanková J, Mejsnarová B. Increased micronuclei frequencies in couples with reproductive failure. Reprod Toxicol. 2000;14:331–5.
33. Lewińska D, Stepnik M, Krajewski W, Arkusz J, Stańczyk M, Wrońska-Nofer T. Increased incidence of micronuclei assessed with the micronucleus assay and the fluorescence in situ hybridization (FISH) technique in peripheral blood lymphocytes of nurses exposed to nitrous oxide. Mutat Res. 2005;581:1–9.
34. Dimitrijević A, Milošević-Djordjević O, Grujičić D, Arsenijević S. Micronucleus frequency in women with genital *Chlamydia trachomatis* infection before and after therapy. Mutat Res. 2006;608:43–8.
35. Iarmarcovai G, Bonassi S, Botta A, Baan RA, Orsière T. Genetic polymorphisms and micronucleus formation: a review of the literature. Mutat Res. 2008;658:215–33.