A Comparative Study for Selectivity of Micronuclei in Cervical Exfoliated Cells on Chronic Boron Effects

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Abstract

**Background:** Cervical cancer is one of the most frequent malignancies in women. Micronucleus (MN) testing has gained popularity as a biomarker in early diagnosis of many types of cancer. **Aims:** This study aims to investigate the role of MN testing on early detection of cervical cancer and the effect of boron exposure on cervical cells. **Settings and Design:** The study population comprised women who were diagnosed to be human papillomavirus (HPV)-positive and had atypical squamous cells of undetermined significance (ASCUS) in a cervical screening project. A total of 15 HPV-positive and 36 ASCUS patients were identified. Randomly selected 20 women were selected from boron-rich region ($n=10$) and nonboron region ($n=10$). **Materials and Methods:** Cervical swab specimens were dyed using Papanicolaou (PAP) and May–Grünwalds–Giemsa (MGG) techniques, and MN count in 1000 cells was performed. The results were statistically evaluated. **Statistical Analysis Used:** Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 13. Quantitative data were presented as mean ± standard deviation. MN test scoring was compared using Mann–Whitney $U$-test. **Results:** Boron content of urine was measured to be $3.02 \pm 1.45$ and $0.98 \pm 0.42$ mg/day in boron-rich and nonboron regions, respectively. When MN counts were compared according to PAP and MGG staining in HPV- and ASCUS-positive women, there was statistically no significant difference ($P > 0.05$). Disregarding regions, HPV/control and HPV/ASCUS cases stained with PAP and MGG had statistically significant difference in MN count ($P < 0.05$). **Conclusion:** These findings suggest that MGG and PAP staining gives similar results with regard to MN count. On the other hand, it has been shown again that HPV induces MN and causes genomic instability. **Keywords:** Boron, May–Grünwalds–Giemsa stain, micronucleus test, Papanicolaou test, uterine cervical neoplasms

**Introduction**

Recent research has shown that boron and its derivatives have preventive and therapeutic potential on various types of cancer. Even though it is essential to therapeutic agents, it is of great importance to establish an early diagnosis of malignancy. A great number of investigations are done on determining methods of early diagnosis and biomarkers. Earlier studies on boron have shown that it may be effective in prostate, breast, lung, and cervical cancer. Recent studies indicate a negative relationship between boron and cancer incidence due to its anticancerogenic effect.[1,2] There is evidence that micronucleus (MN) test may be used for diagnosis and monitoring of prognosis in malignancies. The MN is formed during mitotic division of the cell, is not part of the nucleus, and originates from the chromosome or acentric chromosomal piece.[3] There are study results on the potential of MN testing as a biomarker of early cancer diagnosis.[4–7]

Cervical cancer is the fourth most frequent malignancy in women after breast, colorectal, and lung cancer. Each year approximately 528,000 women are diagnosed with cervical cancer. In 2012, 266,000 lost their lives due to cervical cancer.[8] In Turkey, the incidence of cervical cancer is 4.5 in 100,000 women.[9] Even though there are screening programs for cervical cancer and theoretically it could be prevented, it remains a deadly malignancy especially in developing and underdeveloped countries.

Cervical cancer turns into invasive lesions after passing precancerous stages. Diagnosing cervical cancer in the...
precancerous stage will decrease incidence and mortality.\[^{[10]}\] Screening with Papanicolaou (PAP) test in recent years has demonstrated a decrease in cervical cancer; however, this decrease is not enough. Using PAP and May–Grünwald–Giemsa (MGG) staining besides MN methods may increase the efficiency of screening and diagnostic programs.

This study aimed to evaluate the MN test as a biomarker in human papillomavirus (HPV)-positive and precancerous lesion–positive cases in boron-rich and nonboron regions and to investigate the effect of boron. Another aim was to examine the results of PAP and MGG staining and compare differences with regard to MN induction.

**Materials and Methods**

The boron-rich regions of Turkey are Iskele and Osmanca districts of Balikesir city and the nonboron group consisted of citizens of Çağış district.\[^{[11]}\] A total of 1046 participants of age 30–60 years were included in the study, 534 of which were in the boron group and 512 were in the control group.

Pap smear specimens were sent to Güneş Pathology Laboratory in İzmir, Turkey, as Thin-Prep solutions. Histopathologic evaluation was done using BETHESDA classification. DNA isolation was done by Gene All Ribospin Vrd Kit, and for HPV typing, Seeplex HPV4A ACE Screening kit was used. At the end, 8 HPV(+), 19 atypical squamous cells of undetermined significance (ASCUS), and 10 controls from boron-rich region and 7 HPV(+), 17 ASCUS(+), and 10 controls from nonboron region were evaluated for MN frequency. A total of 71 specimens were included in the study.

The daily boron intake was calculated in 30 women between 30 and 60 years of age living in both boron-rich and nonboron regions by collecting 24-h urine and using creatinine clearance measurement. Creatinine clearance was measured and calculated using 2 mL of blood and urine samples at Balıkesir Sevgi Hospital. Boron measurement was done at Middle East Technical University in Ankara, Turkey, by inductively coupled plasma atomic emission spectroscopy (ICP-OES) technique.\[^{[11]}\]

Boron was measured using ICP-OES device. Solubilization was accomplished using 0.5 mL urine sample using PTFE cups and 5.0 mL HNO\(_3\) in microwave incubator (Milestone Ethos PLUS). Analysis was done using Leeman Labs Inc., DRE model ICP-OES device. For B determination, 249.773 nm was used; for internal standard, In (Indiyum) 230.606 nm wavelength was used.\[^{[11]}\]

After PAP and MGG staining cervical smear specimens, two coded slides were prepared for each subject, and two different people scored a total of 1000 cells from each set of slides. Counts were done using magnification of ×100 (immersion). Cells were isolated on the microscope slides, and if there were cellular degenerations, those were not included. Apoptotic cells and cytoplasmic fragments were excluded. The criteria for scoring were those of Sarto et al. and Tolbert et al.\[^{[12,13]}\]

Ethical approval was obtained from the Faculty of Medicine Local Ethical Committee (18.12.2013 no: 321). Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 13. Quantitative data were presented as mean ± standard deviation. MN test scoring was compared using Mann–Whitney \(U\)-test. \(P\) value <0.05 was considered significant.

**Results**

The mean ages of women from boron-rich and nonboron regions were found to be 45.11 ± 7.96 and 46.88 ± 7.68 years, respectively. Exposure to boron was found to be 41.81 ± 10.73 years, and women from nonboron regions had lived for 41.21 ± 11.79 years in the same place. Body mass indices and age of marriage were found as 29.97±5.62 kg/m\(^2\) and 19.27 ± 2.68 years, respectively, for boron-rich group and 63 ± 4.42 and 19.44 ± 3.85 for nonboron group. None of the volunteers was smoking or using alcohol or taking vitamin/antioxidant supplementations. Prior to boron analysis, renal functions were found to be normal. Urinary boron was calculated to be 3.02 ± 1.45 and 0.98 ± 0.42 mg in boron-rich and nonboron regions, respectively.

Smear specimens were stained by PAP and MGG staining methods, and MN count was done in 1000 cells. Cervical exfoliated cells stained by PAP and MGG are shown in Figure 1 and MN cells are shown in Figures 2 and 3.

There was no difference in MN count between the two regions in virally and pathologically normal specimens \((P > 0.05)\). There was no difference in MN counts between two staining methods in HPV-positive and ASCUS-positive cases \((P > 0.05)\) [Table 1].

Disregarding regions, there was no difference in MN counts between the two staining methods in HPV-positive and ASCUS-positive cases \((P > 0.05)\). As can be seen in Table 2, HPV/control and HPV/ASCUS cases stained with PAP and MGG had statistically significant difference in MN count \((P < 0.05)\). ASCUS/control cases had significant difference for MGG staining \((P < 0.05)\) but not for PAP staining \((P > 0.05)\).

**Discussion**

The main way to prevent cervical cancer is to perform regular cervical cancer screening. In developed countries, the use of the Pap test for nearly 50 years has decreased the incidence of cervical cancer by 70%. However, these results are lower than expected. Additional tests are needed to strengthen the diagnosis. MN assay in our study is a test that allows detection of genomic instability in cells. An increase in the number of MN is considered to be an indirect indicator of numerical and structural chromosome aberrations in cells that form various agents. Several studies in genomic instability

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\[^{[10]}\] Yıldırım, et al.: A comparative study for micronuclei and boron Journal of Cytology \Volume 36 \Issue 2 \April-June 2019
of cancer cells during the progression in terms of increased results were obtained.\textsuperscript{[4,14]}

Comparing MN induction between the two regions, there was no difference between PAP and MGG staining methods. This is show that the amount of boron intake has no effect on the number of MN count. In a study by Korkmaz et al. daily boron intake of 8.41 mg/day was found in boron rich region, however there was no difference in MN count between boron rich and normal region.\textsuperscript{[15]} This result is consistent with the present study data.

### Table 1: Total number of MN in HPV(+) and ASCUS cases in different boron and nonboron regions with different staining methods

| Region       | Subjects (n) | PAP staining average number MN/1000 cells | MGG staining average number MN/1000 cells |
|--------------|-------------|-----------------------------------------|----------------------------------------|
| Boron region |             |                                          |                                        |
| HPV(+)       | 8           | 5.75±3.19                               | 5.37±2.26                              |
| ASCUS        | 19          | 0.95±0.97                                | 2.11±1.88                              |
| Control      | 10          | 0.50±0.70                                | 0.50±0.70                              |
| Nonboron region |         |                                          |                                        |
| HPV(+)       | 7           | 5.86±2.54                               | 4.86±1.95                              |
| ASCUS        | 17          | 2.18±2.46                                | 2.53±2.76                              |
| Control      | 10          | 0.90±0.99                                | 0.60±0.70                              |

MN: Micronucleus; HPV: Human papillomavirus; ASCUS: Atypical squamous cells of undetermined significance; PAP: Papanicolaou; MGG: May-Grünwalds-Giemsa

### Table 2: Comparison of MN counts of PAP- and MGG-stained preparations in HPV/control and HPV/ASCUS case groups regardless of regional differences

| Staining method | Compared groups | P*                |
|-----------------|-----------------|-------------------|
| All regions     | PAP             | HPV/control       | 0.000 |
| All regions     | MGG             | HPV/control       | 0.000 |
| All regions     | PAP             | HPV/ASCUS         | 0.000 |
| All regions     | MGG             | HPV/ASCUS         | 0.000 |
| All regions     | PAP             | ASCUS/control     | 0.177 |
| All regions     | MGG             | ASCUS/control     | 0.001 |

* Mann-Whitney U-test; MN: Micronucleus; PAP: Papanicolaou; MGG: May-Grünwalds-Giemsa; HPV: human papillomavirus; ASCUS: Atypical squamous cells of undetermined significance

In a study conducted by Uçkun et al., the amount of change in the number of MN cells of lymphocytes in individuals living in boron rich region have been identified. Compared with the control group, individuals exposed to boron were found to have lower amounts of MN. The study did not find any genotoxic effects of boron and found that it may have a protective effect against genetic damage.\textsuperscript{[16]}

Compared with healthy subjects, many researchers have observed a high frequency of cells with MN among patients with cancer. MN count is increasing 2.5 or 3.5 times in all patients with cancer when compared with controls.\textsuperscript{[17,18]}

In this study, PAP and MGG staining of HPV-positive women was found to be significantly different with regard to MN count both in boron-rich and nonboron regions. This result shows that HPV genome causes genomic instability in exfoliated cervical cells by a sensitive and reliable test of MN count. Since there was no difference between PAP and MGG staining methods, both may be used to detect genomic instability by MN count. On the other hand, contrary to our study, Palaskar and Jindal compared MN count in buccal exfoliated cells by PAP and MGG staining methods and found that MN count was higher in specimens stained by PAP.\textsuperscript{[19]} This finding concludes that
Yıldırım, et al.: A comparative study for micronuclei and boron

when determining MN frequency, certain criteria should be taken into account.

When regions were disregarded, PAP- and MGG-stained specimens of HPV/control and HPV/ASCUS groups had significantly different MN counts. These results propose that HPV-positive women have genomic instability more than ASCUS and control women. When comparing ASCUS/ control, MGG staining was significantly different; however, PAP staining was not. We may conclude that in women who are HPV-negative but ASCUS-positive, MGG staining may be added to determine genomic instability in a more specific way. ASCUS diagnosis may not be a direct indication of genomic instability. According to the results in ASCUS patients, MN count was more valuable in MGG-stained groups. Samantha et al. stated that MN count in cervical epithelial cells may be used as a biomarker because it is an easy, reliable, repeatable, and objective test. The MN count test is easy and cheap; therefore, it is suitable to be a biomarker for cancer early diagnosis. Our results are in concordance with these studies.

The total HPV and ASCUS total cases in our study showed significantly no difference in MN count between PAP and MGG staining. These results show that MN test is sensitive and reliable for both staining methods. Adding MN count to PAP test to determine genomic instability may be of benefit. In ASCUS-positive cases, MGG staining may be a better choice because it is more sensitive.

Our results show that daily boron intake with exertion of 3.02 ± 1.45 mg/day in urine has no effect on MN incidence. Determination of MN count in specimens stained with PAP is as reliable as those stained with MGG and may be used as a biomarker. It is shown once again that HPV induces MN and causes genomic instability.

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Conflicts of interest
There are no conflicts of interest.

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