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Genotoxic and cytotoxic effects of formic acid on human lymphocytes in vitro

Formik asidin insan lenfositleri üzerindeki in vitro genotoksik ve sitotoksik etkisi

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ABSTRACT

Objective: Formic acid is an ubiquitous chemical constituent in the environment, being produced by sources as diverse as vegetation, ants, soil, vehicles, biomass burning, and photochemical reactions. The present work is focused on in vitro analysis of cytotoxic and genotoxic effects of formic acid, using cytogenetic tests such as the cytokinesis-block micronucleus assay (CBMN) and chromosomal aberration analysis, in human lymphocytes.

Method: This study was carried out using blood samples from healthy, non-smoking adults aged 18-22 years, of whom 10 were male and 6 were famale. Different concentrations (0.07, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 mM) of formic acid was added to the lymphocyte culture test for chromosomal aberration (CA) analysis. Mitomycin-C (0.3 mg/ml) was used as the positive control. Human peripheral blood lymphocyte cells were treated with 20, 40, 60, 80 mM concentrations of formic acid for 48 h. for the CBMN test. Mitomycin-C (0.5 mg/ml) was added to the Lymphocyte culture as a positive control. The present research was carried out to assess the cytotoxic and genotoxic effects of formic acid on human peripheral lymphocytes in vitro using the cytokinesis-block micronucleus assay (CBMN), as well as chromosomal aberration (CA) analysis.

ÖZET

Amaç: Formik asit, bitkiler, arılar, toprak, araçlar, gübre yanması ve fotokimyasal reaksiyonlar gibi çeşitli kaynaklarca üretilen ve çevrede yaygın olarak bulunan kimyasal bir bileşimdir. Mevcut çalışma, insan lenfositlerinde sitokinez-blok mikronükleus tayini ve kromozomal aberasyon analizi gibi sitogenetik testler kullanarak formik asidin sitotoksik ve genotoksik etkilerinin in vitro analizine odaklanmıştır.

Yöntem: Bu çalışma 18-22 yaş arası 10'u erkek, 6'sı kadın sağlıklı, sigara kullanmayan yetişkinlerinden kan örnekleri kullanılarak gerçekleştirilmiştir. Lenfosit kültürüne kromozomal aberasyon (CA) testi için formik asidin farklı konsantrasyonları (0,07, 0,1, 0,2, 0,3, 0,4, 0,5, 0,8 mM) eklendi. Pozitif kontrol olarak mitomycin-C (0,3 µg/ml) kullanıldı. Sitokinez-blok mikronükleus tayini (CBMN) için, insan periferik kan lenfositleri 20, 40, 60, 80 mM derişimde formik asitle 48 saat işleme tabi tutuldu. Lenfosit kültürüne, pozitif kontrol olarak mitomycin-C (0,50 µg/ml) eklendi. Bu araştırma, sitokinez-blok mikronükleus tayinini (CBMN) ve kromozomal aberasyon (CA) analizini kullanarak in vitro insan periferal lenfositlerinde formik asidin sitotoksik ve genotoksik etkilerini değerlendirmek için yapılmıştır.

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Results: A significant increase was observed for induction of micronucleus frequency in all treatments of formic acid concentrations for 48 h. comparing with the negative control and mitomycin C (MMC, 0.5 µg/ml) which was used as positive control. When compared with negative control and with mitomycin C (MMC, 0.3 µg/ml) which is used as positive control, it is observed that formic acid is rising the frequency of chromosomal aberration significantly at all appliance concantrations in 24 h. The frequency of micronuclei and chromosomal aberrations increased in a dose dependent manner. The results showed that there were significant correlations between formic acid concentration and micronuclei frequency (r= 0.92), numbers of necrotic cells (r= 0.95), and apoptotic cells (r= 0.91).

Conclusion: Our data provided evidence that there is a significant correlation between the concentration of formic acid and the following chromosomal aberrations: frequency of micronuclei, apoptotic cells, and necrotic cells in vitro.

Key Words: Formic acid, human lymphocyte culture, chromosomal aberration, micronucleus, cytotoxicity

Bulgular: Pozitif kontrol olarak kullanılan mitomisin C (MMC, 0,5 µg/ml) ile negatif kontrol karşılaştırıldığında formik asitin tüm dozları mikronükleus frekansını belirgin bir düzeyde arttırdığı gözlenmiştir. Pozitif kontrol olarak kullanılan mitomisin C (MMC, 0,3 µg/ml)'nın negatif kontolle karşılaştırıldığında formik asidin 24 saatte tüm uygulama derişimlerinde, kromozomal aberasyonların sıklığını belirgin bir şekilde arttırdığı gözlenmiştir. Mikronükleus sıklığı ve kromozomal aberasyonlar, doza bağımlı olarak artmıştır. Sonuçlar formik asit konsantrasyonu, mikronükleus frekansı (r=0,92), nekrotik hücrelerin sayısı (r=0,95) ve apoptotik hücreler (r=0,91) arasında anlamlı korelasyonların var olduğunu göstermektedir.

Sonuç: Verilerimiz, in vitro olarak formik asit konsantrasyonu ve mikronükleus frekansı, apoptotik hücreler ile nekrotik hücreler gibi kromozomal aberasyonlar arasında anlamlı bir korelasyon olduğu kanıtını sağlamıştır.

Anahtar Kelimeler: Formik asit, insan lenfosit kültürü, kromozomal aberasyon, mikronükleus, sitotoksisite

INTRODUCTION

Formic acid is the smallest member of the family of saturated monocarboxylic acids; it is a colorless liquid with a tangy odor and a density of 1.22 gram/cm^3. It dissolves in water, alcohol, and ether at all proportions. It burns when it comes in contact with skin. If it is heated to 160 °C, it decomposes into carbon dioxide and hydrogen. It exists freely in nature in wood tar, nettles, ants, perspiration, urine and bouillon. Formic acid pollutes water and can spread through the air. Methyl alcohol is transformed into formaldehyde and formic acid by the body's metabolism; its toxicity is due to the acidosis resulting from the formic acid. As a result of this acidosis, nerve damage to the retina and, depending upon the degree of severity, blindness and eventually death may occur (1, 2). Most of the formic acid in tissues is oxidized via a tetrahydrofolic acid-dependent pathway to CO_2 and H_2O, such as in the liver, erythrocytes and kidneys (2). Literature on the carcinogenic and mutagenic effects of formic acid is quite limited. Tests of sister-chromatid exchange (SCE) performed on the typhimurium strains TA100, TA1535, TA97 and TA98 of *Salmonella* did not find it to be mutagenic. However, mutagenicity was reported in a test conducted on induction of sex-linked recessive lethal mutations as well as another study conducted on chromosomal aberrations (3, 4).

The present work is focused on in vitro analysis of cytotoxic and genotoxic effects of formic acid, using cytogenetic tests such as the cytokinesis-block micronucleus assay and chromosomal aberration analysis in human lymphocytes.

MATERIALS and METHODS

This study was carried out using blood samples from healthy, non-smoking adults aged 18–22 years, of whom 10 were male and 6 were famale. Donors provided written, informed consent at the time of donation for the use of their blood samples. Heparinized whole blood (0.4 ml) was added to 5 ml chromosome medium (Biochrome). Cultures were incubated at 37 °C for 72 h. Human lymphocytes were then exposed to different concentrations (0.07, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 mM) of formic acid for 24 h. A positive control (mitomycin-C), 0.3 µg/ml) was included in every experiment (5, 6). Lymphocytes were cultured in the dark for 72 h, and metaphases were blocked during the last 2 h with colchicine at final a concentration of 0.06 µg/ml. Cells were collected by centrifuging (377 g, 10 min) and resuspended in a hypotonic KCl solution (0.075 M) for 30 min at 37 °C. At the end of this procedure, cells were centrifuged again and fixed in a cold methanol:acetic acid (3:1) mixture for 35 min at +4 °C. Following this process, cells were treated two times with fixative. Slides were then prepared by dropping concentrated cell suspensions onto the glass, followed by air drying. The air-dried slides were stained for 15–20 minutes with 5% Giemsa stain (pH 6.8) prepared in a Sorensen buffer. One hundred metaphases per culture were analysed for the presence of chromosomal aberrations (CA). The number of CAs was obtained by calculating the percentage of metaphases at each concentration and treatment period that showed structural or numerical chromosome aberrations. Chromatid and chromosome breaks, chromosome exchange and chromatid unions, and polyploid cells were screened at all treatment concentrations (5, 7)

For the micronucleus test in cultured human lymphocytes (CBMN), blood samples were added to 5 ml of chromosome medium (Biochrome). Cell cultures were incubated at 37 °C for 72 h. Cytocalasin-B (6 µg/ml) was added to arrest cytokinesis at 44 h after culture initiation. Human lymphocytes were treated with different concentrations (20, 40, 60 and 80 mM) of formic acid for the CBMN test. Cells were exposed to the chemical for 48 h and harvested by centrifuging (167 g, 10 min), and then the pellets were resuspended in a hypotonic solution of 0.075 M KCl for 5 min at +4 °C. The cells were centrifuged again and fixed in a cold methanol:acetic acid (3:1) mixture for 15 min. The fixation procedure was administered three times. Formaldehyde (1%) was added to the last fixative to preserve the cytoplasm. Slides were prepared by dropping concentrated cell suspensions onto the glass, followed by air drying. For CBMN analysis, staining was performed using 5% Giemsa (pH = 6.8) prepared in a Sorensen buffer solution for 20–25 min. Slides were then washed in distilled water and dried at room temperature. Positive control (mitomycin-C (MMC), 0.50 µg/ml) was also maintained in the CBMN experiment (8).

Micronuclei were scored in 2000 binucleate lymphocytes for each subject (9). The nuclear division index (NDI) was evaluated using the following formula: NDI = (M1 + 2(M2) + 3(M3) + 4(M4))/N, where M1–M4 indicates the number of cells with 1 to 4 nuclei, and N indicate the total number of cells scored. The NDI of each cytochalasin B-treated culture was determined by screening 2000 interphase cells for the number of nuclei they contained (10).

Apoptotic and necrotic cells were identified with light microscopy according to morphological characteristics of the nucleus. In order to differentiate apoptotic cells from necrotic cells, we checked for the properties of necrotic cells, which exhibit a pale cytoplasm or loss of cytoplasm, numerous vacuoles, and a damaged/irregular nuclear membrane with a partially intact nuclear structure (9). 2000 cells were counted from each sample. The nuclear division cytotoxicity index (NDCI) = (Ap+Nec + M1 + 2(M2) + 3(M3) + 4(M4))/N was evaluated according to Fenech (9), where Ap = the number of apoptotic cells, Nec = the number of necrotic cells, M1–M4 = the number of viable cells with 1–4 nuclei and N = the total number of cells scored.

Statistical analyses of data were done using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego California USA). Chromosomal aberration frequencies in the cell cultures were analysed using Fisher's exact test. CBMN data were statistically analysed using the F-test for analysis of variance (ANOVA). The significance of differences between the negative control and the series of treatment groups were compared with Dunnett's t-test.

RESULTS

Compared to the other groups, an insignificant number of chromosomal aberrations was found in the metaphases obtained from the blood samples taken from the negative control group where no formic acid was added to the culture (Fig. 1A). Formic acid was administered to parallel cultures at doses of 0.8 mM and lower (0.07, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 mM). It was observed that division stopped fully in the culture where the number of metaphases was very low, and as a result the mitotic index was significantly decreased. After a trial was performed, it was determined that an activation period of 24 hours was suitable and that a longer period would prevent mitotic division. It was determined that the chromosomes condensed in the metaphases appeared filose and had a banded appearance (Fig. 1B). It was further observed that inhibition of cell division continued, but chromosomal anomalies in the metaphases were better visualized at an administered dose of 0.5 mM formic acid (Fig. 1C). Banding in chromosomes and chromosome combinations were then observed more clearly. After treatment with 0.4 mM formic acid, it was observed that mitosis was inhibited and that there were breaks in the chromatids and chromosomes in the metaphases (Fig. 1D).

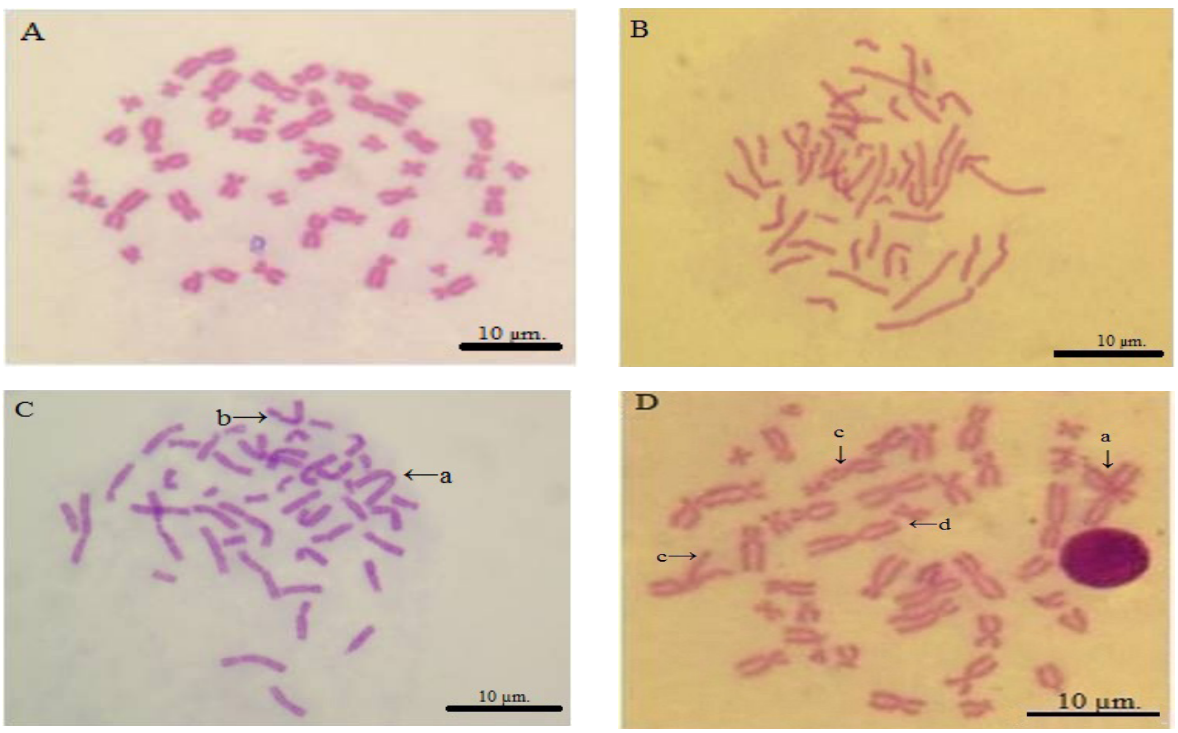

Figure 1. **A.** Metaphase plaque obtained from a normal individual. **B.** Metaphase plaque obtained from the culture that was administered 0.8 mM of formic acid. **C.** Metaphase plaque obtained from the culture that was administered 0.5 mM of formic acid: **a.** Chromosome combination, **b.** Chromosome break **D.** Metaphase plaque obtained from the culture that was administered 0.4 mM of formic acid: **a.** Chromosome combination, **c.** Chromatide break, **d.** Chromatide combination. (×1000, Bar: 10 µm).

A significant increase in the frequency of chromosomal aberrations was observed in some treatments with formic acid after 24 h, compared to the negative control or treatment with mitomycin C, which was used as a positive control. The results obtained after treatment with 0.07 mM formic acid were in line with the results obtained with the negative control. Polyploidy was observed at some formic acid treatment doses (0.3, 0.4 mM) and after treatment in the positive control (Table1).

Cytokinesis events were prevented by cytochalasin-B, and the frequencies of micronuclei and cell death (apoptosis and necrosis) were examined. Formic acid significantly increased the frequency of micronuclei in a dose dependent manner (r = 0.92). The nuclear division index (NDI) was calculated according to the number of nuclei present in the cells. Table 2 demonstrates that the NDI and nuclear division cytotoxicity index (NDCI) were significantly influenced by formic acid.

Table 1. Chromosome anomalies in human peripheral lymphocytes exposed formic acid for 24 hours

Test substance	Treatment		Structural aberrations				Numerical aberrations	Frequency of aberrant cell±SEM (%)	Mitotic index ±SEM (%)
	Period (h)	Doses	ctb	csb	cse	cu	p		
NC	24	1	1	1	-	-	-	0.5±0.2	4.3±0.3
MMC (µg/ml)	24	0.3	19	19	3	7	1	14.5±3.8*	1.7±1.2*
Formic acid (mM)	24	0.07	2	2	-	2	-	1.5±0.5	4.3±1.2
		0.1	3	3	1	4	-	2.7±0.6	4.1±0.8
		0.2	4	4	2	7	-	4.2±1.0	4.0±1.0
		0.3	7	7	2	13	1	9.7±4.5	3.8±0.9*
		0.4	11	11	1	15	1	9.5±2.9	3.5±0.5*
		0.5	12	12	3	18	-	11.3±3.0*	2.8±0.8*

ctb: chromatid break, csb: chromosome break, cu: chromatid union, cse: chromosome exchange, nc: negative control (%1 distilled water), MMC: (0.3 µg/ml mitomycine-C (24 hours), p:polyploidy.
* p<0.05 as compared to control. Fisher's Exact Test.

Table 2. The effects of formic acid on micronucleus frequency, nuclear division index, and nuclear cytotoxic division index in human lymphocytes cultures

	Administration		Number of Cells Counted	Distribution of MN number into Binuclear cells				MN/cell (%)±SEM	NDI	NCDI
	Time (s)	Dose		1	2	3	4			
Negative Control	-	-	2000	1	0	0	0	0.002±0.25	1.981±0.34	1.981±0.25
PC (MMC)	48	0.5 µg/ml	2000	150	16	1	0	0.40±36.2	1.332±0.83	1.359±1.10
Formic acid	48	20 mM	2000	7	1	1	1	0.02±1.50	1.713±0.38*	1.731±0.48
	48	40 mM	2000	7	1	1	2	0.02±1.40	1.678±0.55*	1.697±0.76
	48	60 mM	2000	9	3	2	2	0.04±1.63	1.626±0.68*	1.667±0.47
	48	80 mM	2000	17	2	1	1	0.05±3.95	1.592±0.78*	1.625±0.55

*Significantly different from the negative control P < 0.05 (Dunnett's t-test). NDI: Nuclear division index, NCDI: Nuclear cytotoxic division index, SEM: Standard error of the mean.

NDI and NDCI values were lower than in controls. The lowest NDI values were observed in cultures treated with 80 mM formic acid. All doses of formic acid administered increased micronuclei frequency and cell death to a statistically significant degree ($p<0.05$) compared with the negative control group (Fig. 2A-G). There was a positive correlation between MN frequency, the number of apoptotic cells ($r=0.91$), and the number of necrotic cells ($r=0.95$) (Fig. 3A-C).

DISCUSSION

One of the most sensitive methods used to determine the genotoxic risks of mutagens and carcinogens is assessing the frequency of chromosome aberration (CA) in peripheral blood lymphocytes (11, 12). Chromosome aberrations occur due to damage at the DNA level. For instance, chromosome breaks may be caused by unrepaired double chain breaks in the DNA, and the emergence of chromosomes with a new structure may be caused by the false repair of chain breaks in the DNA (13). Due to the fact that the mechanisms by which chromosomal anomalies emergence resemble each another in different tissues, the level of anomalies in lymphocytes is considered to be an indication of the level of anomalies in other tissues that tend to be cancerous; thus it is also an indicator of cancer risk (14, 15). A high frequency of chromosomal aberrations may give an advance indication that there is a high risk of cancer, regardless of the reason that the increase in chromosome anomalies was triggered. Anomalies of both the chromatid type and chromosome type are indicators of cancer risk. However, there is evidence that chromosome anomalies are better determinants than chromatid anomalies (15, 16).

Another type of cytogenetic method used to determine genotoxicity and carcinogenicity is the micronucleus (MN) test (17, 18). According to Bonassi et al. (19), a high MN frequency in

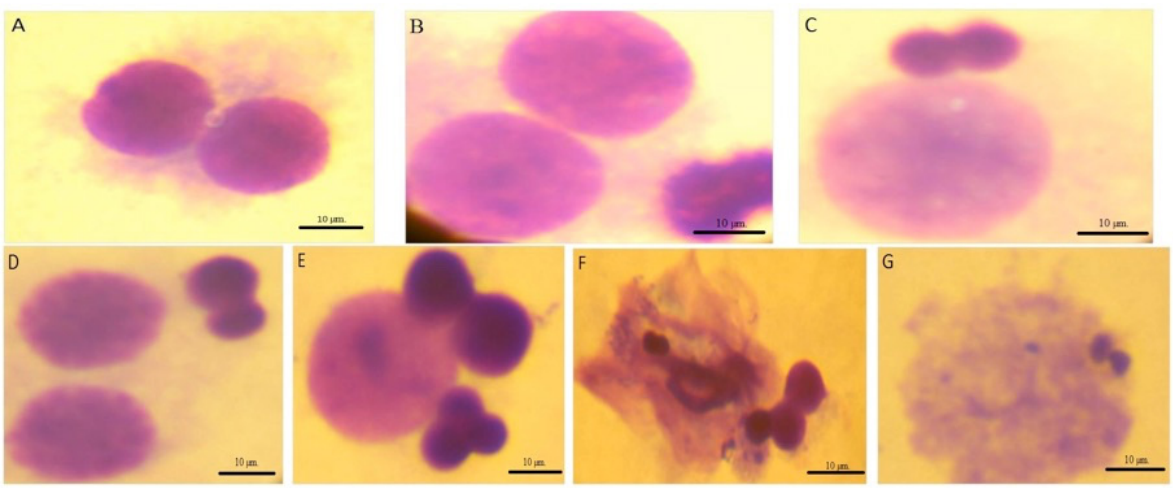

Figure 2. Micronucleus creations lymphocyte culture which was administered different doses of formic acid A. Double nucleus cell in which the normal cytokinesis was inhibited. B. Double nucleus cell containing one micronucleus the cytokinesis of which was inhibited (20 mM formic acid, process of 48 hours). C. Single nucleus cell containing two micronuclei in which the cytokinesis was inhibited (40 mM formic acid, process of 48 hours). D. Double nucleus cell containing two micronuclei in which the cytokinesis was inhibited (60 mM formic acid, process of 48 hours). E. A cell with multi micronuclei in which the cytokinesis was inhibited and which is entering apoptosis (80 mM formic acid, process of 48 hours). F. An apoptotic cell (80 mM formic acid, process of 48 hours). G. Necrotic cell with a pale cytoplasm, many small vacuoles and a cytoplasmic and nuclear membrane with corrupted structure (80 mM formic acid, process of 48 hours). (×1000, Bar: 10 µm).

peripheral blood lymphocytes indicates a risk of cancer in humans. When the cytokinesis blockage micronucleus method was developed by Fenech and Morley (20), researchers began to examine the MN in cells which have completed nuclear division. MN are the small nuclei found apart from their sister nucleus, created during telophase due to the breaks in non-centric chromosomes or chromatid, and to the backwardness of all chromosomes or chromatides (slow chromosomes) (21). In a study that investigated the cytogenetic effect using the micronucleus, chromosomal aberration and sister chromatid exchange method, mercuric chloride has been shown to be a clastogenic chemical because of the variety of chromosomal abnormalities and the increase in the number of micronucleated cells (22).

Formic acid is used in carbonated drinks, fruit, vegetables and canned food as a preservative substance. Furthermore, it is commonly employed by honey producers to combat parasitic Varroa destructor mites in bee colonies (23). In one of their studies, Morita et al. tested the relationship between the clastogenic activities

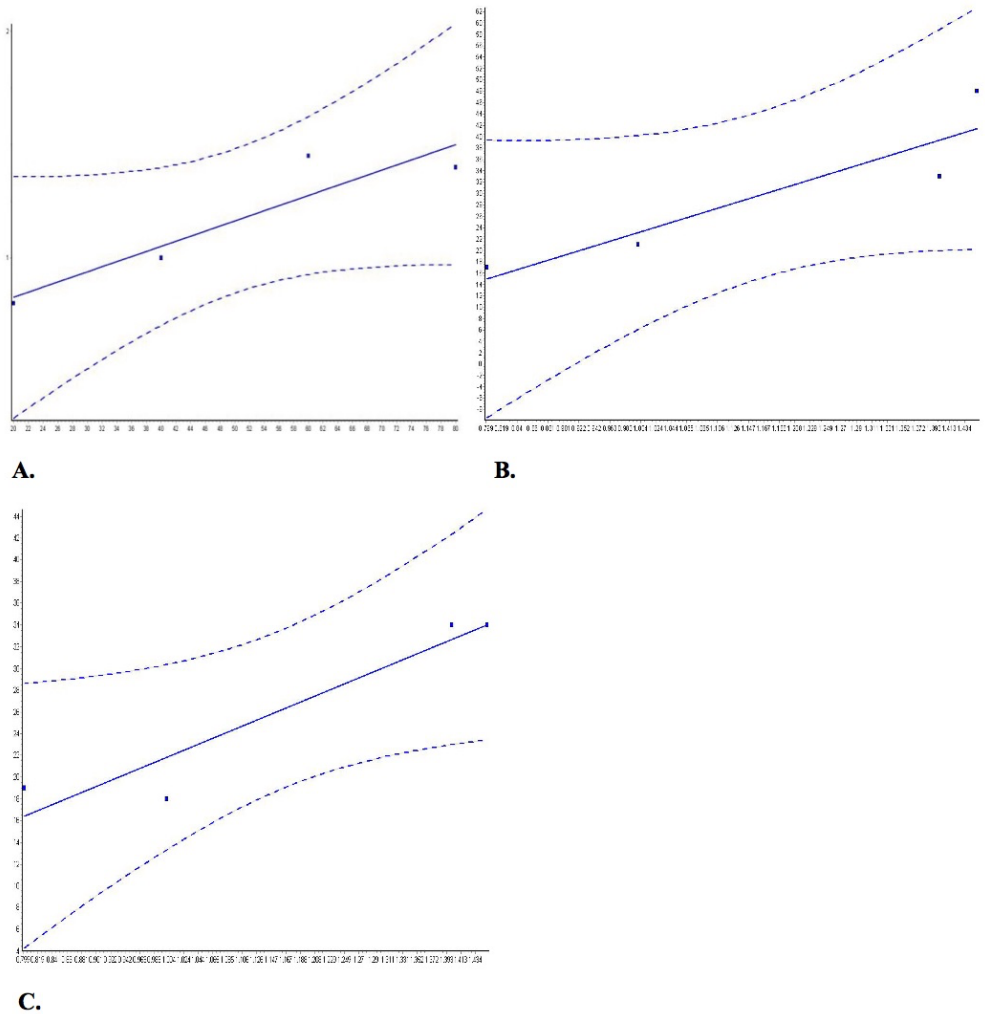

Figure 3. A. Formic acid dose-micronucleus regression charts. **B.** MN frequency-apoptotic cell regression charts (r=0.91, p<0.05). **C.** Micronucleus frequency-necrotic cell regression charts (r=0.95, p<0.05).

of formic acid and the pH of the medium using Chinese hamster ovary K1 cells. That study reported that acids stimulated chromosomal aberrations in the media with an original pH of 6. It was also determined that 12-16 mM concentrations of this acid exhibited toxicity in the media at pH 5.7 or lower (3). In a study of the inhibitory effect of a mixture of propionic acid and formic acid on *Salmonella* pullorum, it was reported that the mixture of propionic acid-formic acid caused a significant decrease in the number of colonies in the culture compared to the control (24). DNA adductions formed by various chemicals play an important role in cancer initiation. Wang et al. (25) reported that formic acid caused DNA adducts as well as hemoglobin (Hb) adducts in mice.

The frequency of micronuclei in peripheral blood lymphocytes is used as a biomarker for chromosome damage (9). The doses that were used in the MN and chromosomal aberration studies were different. The chromosomal aberration treatment dose is typically lower than the MN dose (26-28). However, in some studies the dose producing MN was found to be equal to the dose causing chromosomal aberrations (29, 30). In our study, the dose that caused chromosomal aberrations was found to be lower than that which leads to micronuclei formation.

Conclusion: Our data provide evidence that there is a significant correlation between the concentration of formic acid and the following chromosomal aberrations: frequency of micronuclei, apoptotic cells and necrotic cells in vitro.

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CONFLICTS of INTEREST

The authors declare no conflicts of interest.

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