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Genotoxicity of a synthetic plant growth regulator, Forchlorfenuron (CPPU), on human lymphocytes using chromosome aberration assay

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Abstract. Forchlorfenuron (FCF, also known as CPPU), which belongs to the group of phenylurea cytokinins, is one of the most widely used synthetic plant growth regulators (PGRs) worldwide. Although FCF plays a crucial role in cellular growth and differentiation by promoting cell division in plants, it disrupts higher-order septin assembly in other eukaryotic organisms, including humans. Despite its widespread use, no study has been found investigating the genotoxic effects of this synthetic PGR on humans. Hence, this investigation was designed to examine the potential cyto-genotoxicity of a commercial formulation of FCF on human peripheral blood lymphocytes (PBLs) using chromosome aberrations (CAs) and mitotic index (MI) endpoints. The whole blood cultures were treated with 0.25, 0.50, 1.00, and 2.00 μg/ml concentrations of a commercial form of FCF. According to the results, FCF significantly enhanced the percentage of cells containing structural CAs at the concentrations of 1.00 and 2.00 μg/ml for both treatment times (24 and 48 h), in comparison to the negative control ($P<0.05$). Besides, in cultures exposed to FCF concentrations of 0.50, 1.00, and 2.00 μg/ml, the total CA/cell ratio was significantly higher ($P<0.05$). In addition, FCF was found to have cytotoxic activity on human PBLs at all treatments (except for the lowest concentration at 24 h) by significantly reducing the MI compared to the negative control ($P<0.05$). The findings of this investigation indicate the first time that a commercial formulation of FCF (0.50-2.00 μg/ml) may have genotoxic and cytotoxic potential on human lymphocytes.

Keywords: Forchlorfenuron, CPPU, synthetic plant growth regulator, chromosome aberration, mitotic index, human lymphocytes.

INTRODUCTION

Today, environmental pollutants and their toxic effects on organisms have become one of the most discussed issues worldwide (Briggs 2003; Zhang et al. 2011). The main pollutant groups that cause environmental pollution are chemicals used in agriculture such as herbicides, insecticides, fun-

gicides, and plant growth regulators (PGRs), which are an important part of modern agriculture (Lu et al. 2015; Wang and Yang 2016; Rodrigues et al. 2018).

Synthetic PGRs also known as environmental hormones are a class of agrochemicals that stimulate plant developmental processes such as organ formation, cell division, and growth (Wang et al. 2011). People are potentially exposed to PGRs either directly as workers in greenhouses and agriculture or indirectly through food consumption. Therefore, the uncontrolled and excessive use of PGRs both cause environmental pollution and threaten non-target organisms and ultimately human health. Gangadhar et al. (2020) reported that residues of synthetic PGRs can seriously harm human health.

Forchlorfenuron (FCF, also known as CPPU) which is included in the phenylurea synthetic cytokinin group, is among the most widely used PGRs worldwide to increase fruit size/enlargement (USEPA 2004; Heasley et al. 2014). It is known that synthetic cytokinins (i.e. FCF, thiadizuron, and their derivatives) are cytokinin oxidase/dehydrogenase (CKX) inhibitors. The catabolic cytokinin dehydrogenase CKX, is bound by FCF and is competitively inhibited, leading to an increase in intracellular cytokinin levels and, eventually, larger fruits (Kopecný et al. 2010; Heasley et al. 2014). It was also suggested that FCF promotes plant cell division and lateral growth through its synergistic interactions with endogenous auxins, thereby inducing parthenocarpy, which in turn increases fruit size, fruit set, and fruit cluster weight (USEPA 2004; Kim et al. 2006; Su et al. 2021). It is widely used to enlarge many fruits, such as grapes, apples, and kiwi (Kim et al. 2006; EFSA 2017).

Unfortunately, in 2011, FCF was abused by Chinese farmers to grow larger watermelons; but some of them exploded. After this event, the potential threats it poses for organisms and the environment have started to come to the fore more (Gong et al. 2021). It has been reported that FCF and its metabolite residues have frequently been found in fruits, water, sediments, and aquatic species (Meng et al. 2020; Gong et al. 2021). In addition, FCF is bioaccumulated by humans and can reach concentrations that may be harmful to farm laborers (Shi et al. 2012; Toumi et al. 2018). Tixier et al. (2001) indicated that FCF may be an endocrine disruptor and had certain genotoxic and ecotoxic effects. In a recent study also reported the excessive use of swelling agents such as FCF, and thidiazuron in fruits and vegetables has caused food safety problems (Wang et al. 2023). However, the use of FCF in seedless fruit formation by inducing parthenocarpy is still common (Su et al. 2021).

While FCF has been used as a PGR in many countries, it has also been an experimental molecule extensively used to investigate septin functions (Angelis et al. 2014). Septins, a class of GTP-binding proteins, are found in all eukaryotic organisms with the exception of higher plants, and have many important cellular functions (Henzi et al. 2021). It has been reported that FCF disrupts septin localization in budding yeast and causes defects in cytokinesis (Iwase et al. 2004). The researchers showed the FCF causes the accumulation of thick septin bundles and aggregates by blocking septin filament turnover (Hu et al. 2008).

The stability of the septin cytoskeleton induced by FCF has been shown to mimic the functional effects of septin depletion, such as inhibition of cell division, cell-cell adhesions, calcium ingress, migration, and vesicle trafficking (Hu et al. 2008; Sidhaye et al. 2011; Sharma et al. 2013; Tokhtaeva et al. 2015; Marcus et al. 2016; Zhang et al. 2016). The stabilization of septin filaments by FCF impairs their normal functions (Hu et al. 2008). Because FCF influences different cellular systems via stabilizing septins in metazoans, it appears crucial to look into its potential to be genotoxic to humans.

According to our literature search, only one study was found regarding the genotoxic potential of FCF. It was reported that FCF was not mutagenic using the mouse bone marrow micronucleus test (Lin et al. 2012). However, no studies were found to investigate the genotoxic risks of FCF on humans. Therefore, we believe that it is crucial to identify the potential genotoxic/cytotoxic risks of the commercial formulation of FCF (sitofex, active ingredient 10 g/l FCF), which is a widely used synthetic PGR today.

Hence, this study was carried out to investigate the genotoxic and cytotoxic potential of FCF, in a commercial form, using *in vitro* chromosomal aberration (CA) and mitotic index (MI) assays, on human peripheral blood lymphocytes (PBLs).

MATERIALS AND METHODS

Test samples and chemicals

Samples of whole blood were provided from four healthy, non-smoker, and non-alcoholic different donors (30-32 years old, 2 females, and 2 males). Donors were selected from people who did not use drugs in the last three months and were not exposed to environmental pollutants such as pesticides, dust, and chemicals at a high rate.

This research was carried out in accordance with the Helsinki Declaration and ethical approval has been given from the Institutional Ethics Committee of Hatay Mustafa Kemal University, Türkiye (approval number:

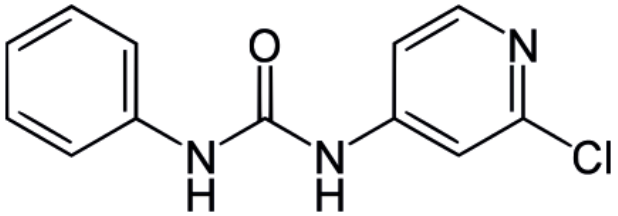

Figure 1. The chemical structure of Forchlorfenuron.

14.02.2019/09). In addition, each participant signed a permission form and provided their informed consent to take part in the study.

A commercial formulation of FCF (Sitofex, containing 10 g/L FCF as the active ingredient) was obtained from the Agrikem company, Türkiye. Its properties are as follows: IUPAC name: 1-(2-chloro-4-pyridyl)- 3-phenylurea; CAS name: N-(2-chloro-4-pyridinyl)-N-phenylurea; Trade names: CPPU, KT-30; Chemical formula: $C_{12}H_{10}ClN_3O$; Molecular weight: 247.68 g/mol; CAS registry number: 68157-60-8. The chemical structure of FCF is given in Figure1. Mitomycin-C (MMC, M-05030), and Colchicine (C-9754, St. Louis, MO) were supplied from Sigma. The other chemicals used in the study were provided by Merck. (Darmstadt, Germany). As the culture media, chromosome medium B (Biochrome F-5023; Berlin, Germany), was used.

Chromosome Aberration (CA) assay

The CA assay was performed according to the protocol of Evans (1984) with minor adjustments (Kocaman and Topaktaş 2007). Heparinized whole blood samples (0.2 ml) obtained from each donor were added to chromosome medium B (2.5 ml) to set up the cultures, which were then incubated at 37 °C for 72 h. The components of the culture medium were (amounts per liter) as follows: minimum essential medium (MEM-joklik) with non-essential amino acids (850 ml), fetal bovine serum (150 ml), heparin (25000 U), penicillin G, sodium salt (75000 U), streptomycin sulfate (50 mg), phytohemagglutinin L (2.5 mg), ascorbic acid (5.0 mg), and glutathione (reduced) (5.0 mg). The commercial FCF formulation was applied to the cultures at concentrations of 0.25, 0.50, 1.00, and 2.00 µg/ml. The highest concentration of FCF (2.00 µg/ml) was determined according to the half-maximal inhibitory concentration (IC_{50}) value that reduced MI by approximately 50% compared to the negative control. The commercial FCF used in this study was in liquid form and its serial dilutions were prepared freshly and under sterile conditions before each experiment by diluting with sterile distilled water (based on the amount of active ingredient it contains). Treatment times were conducted as 24 and 48 h. Parallel tests were also conducted using a negative (untreated) and positive (treated with 0.2 µg/ml MMC) control.

Two hours prior to harvesting, colchicine, at a concentration of 0.06 µg/ml, was given to culture tubes containing the cells. At the 72nd h, the cultures were centrifuged for 5 min at 2000 rpm and the supernatant was removed. The pellets were treated gently with the hypotonic solution (0.4% KCl, at 37 °C) for 15 min. Thereafter, the suspension was centrifuged at 1200 rpm for 10 min and the cells in pellets were fixed three times with a cold fixative (3:1 v/v; methanol: glacial acetic acid, at 22 °C) for 20 min. Finally, slides were prepared by dropping 3-4 drops of the final cell solution on clean, chilled glass slides, and standard procedures were used to stain the slides (5% Giemsa in Sorensen Buffer, for 15-20 min at pH = 6.8), after air-drying.

Microscopic evaluation

In this research, the slides were examined using an Olympus CX21 light microscope at 1000x magnification for CAs, and also 400x magnification for MI. The CAs observed in this study were categorized as structural and numerical (polyploid cells) aberrations according to Mosesso et al. (2013) and Ayabaktı and Kocaman (2020). By considering the same references, structural CAs were further grouped into chromatid (breaks, sister unions, exchanges), and chromosome types (breaks, dicentrics, fragments). In order to score the CAs, a total of 400 metaphases were examined per concentration, for each treatment and donor. Following the scoring of CAs, the percentage of cells having structural CAs, the total number of CAs per cell, as well as the percentage of frequency of each type of CA, were computed.

To calculate MI, the percentage of metaphases within 2000 cells was counted per culture for each treatment and donor (i.e., 8000 cells in total per concentration) using following equation:

$$MI = 100 \times \text{cells in metaphase} / 2000$$

Statistical analysis

For statistical analysis, an experimental unit of four subjects (n=4) was employed. Data were displayed as mean ± standard deviation (SD). The statistical analysis was conducted by utilizing One-Way Analysis of Variance (ANOVA). To compare the experimental groups, a

post-hoc analysis (least significant difference; LSD) test was performed. Relationships between concentration and response were evaluated by correlation and regression coefficients (r^2). The results were considered statistically significant according to the significance level of $P<0.05$.

RESULTS

The effects of the commercial formulation of FCF on human lymphocytes by induced CA are presented in Table 1. According to the results obtained from the *in vitro* CA test; FCF significantly increased the percentage (%) of cells with structural CAs at 1.00 and 2.00 µg/ml for both treatments, 24 and 48 h, in comparison to the negative control ($P<0.05$). Additionally, it was determined that FCF significantly increased total CA/cell at three high concentrations (0.50, 1.00, and 2.00 µg/ml) for both exposure times (24 and 48 h) compared to the negative control ($P<0.05$). However, CA formations caused by FCF in this study were not as effective as the MMC, positive control, and were determined to be significantly lower compared to the MMC at all concentrations and treatment times ($P<0.001$).

Regression analysis results revealed that the percentage of cells including structural CA showed a concentration-dependent increase in just 24 h treatment (for 24 h: $r^2= 0.837$, $P<0.05$ and for 48 h: $r^2= 0.727$, $P>0.05$). Similarly, during the 24 h treatment period, there was a concentration-dependent rise in the frequency of total CAs/cell (for 24 h: $r^2= 0.897$, $P<0.05$ and for 48 h: $r^2= 0.733$, $P>0.05$).

In our study, it was determined that FCF caused especially structural CAs in cultured human lymphocytes. Chromatid breaks (63.07%) (Fig. 2a) and sister chromatid unions (29.41%) (Fig. 2b) were the most frequent chromatid-type structural CAs found in PBLs treated with FCF. However, chromatid exchange (0.65%) (Fig. 2c) was found at a low frequency. In addition, chromosomal type structural CAs caused by FCF were observed at low frequencies as chromosome break (4.25%) (Fig. 2d) and dicentric chromosome (0.33%) (Fig. 2e). As shown in Table 1, in this investigation, only polyploid cells were observed, albeit at a low frequency (2.29%) (Fig. 2f), in the form of numerical CA.

In the present research, MI (percentage of cells in mitosis) values were determined to assess the possible cytotoxic effect of FCF (Table 2). In comparison to the negative control, FCF generally led to a significant decrease in MI for all concentrations (0.25, 0.50, 1.00, and 2.00 µg/ml) and both treatment durations (24 and 48 h) (except for the lowest concentration, 0.25 µg/ml, for 24 h) ($P<0.001$). Although FCF caused decreases in the MI values as the concentration increased; there were no statistically significant concentration-effect relationships ($P>0.05$).

DISCUSSION

Forchlorfenuron is a synthetic phenylurea-derived cytokinin widely used in agriculture as one of the PGRs which regulates the growth and development of plants (USEPA 2004; Heasley et al. 2014). Despite its widespread use, no available studies were found on the genotoxicity and cytotoxicity of FCF on human cells. Hence, the geno/cytotoxic effects of cytofex, a commercial form of PGR containing FCF as the active ingredient, on human PBLs cultures at four distinct concentrations (0.25, 0.50, 1.00, and 2.00 µg/ml) and two exposure times (24 and 48 h) were evaluated using *in vitro* CA test. Evaluation of the increase in CA frequency is an important biomarker of the genotoxic potential and allows the early detection of substances that cause damage to DNA (Bonassi et al. 2008; Murgia et al. 2008). Due to the human lymphocytes being primary cells and having a low spontaneous rate of chromosomal damage they are preferred in *in vitro* studies (Phillips and Arlt 2009).

The findings of the study suggested that FCF could be genotoxic since it markedly enhanced the CA formation in human PBLs. The fact that the percentage of structural CAs significantly increased following exposure to high concentrations (1.00 and 2.00 µg/ml) of FCF supports the clastogenic action of this compound by causing DNA strand breaks. In addition, in cultures treated with FCF, a few polyploid cells were found as an indicator of the aneugenic effect. However, even though at a low frequency, it should not be overlooked that these numerical abnormalities contribute to the increased overall genotoxic effect caused by FCF. As can be seen in Table 1, when evaluated in terms of total CA per cell, the cultures exposed to 0.50 µg/ml of FCF were also found genotoxic. That is, as compared to the negative control, FCF was found to significantly induce the proportion of total CAs/cell at three concentrations (0.50, 1.00, and 2.00 µg/ml), thereby potentially genotoxic to human PBLs.

Contrary to the results of our study, it was reported that an oral LD_{50} dose of FCF 568 mg/kg in male mice and 421 mg/kg in female mice didn't show a mutagenic effect using a mouse bone marrow micronucleus assay (Lin et al. 2012). Bu et al. (2019) reported a study on the effect of FCF on ovarian function of Sprague-Dawley rats. In that chronic toxicity study, Sprague-Dawley rats were fed FCF at dosage levels of 0, 0.6, and 60 mg/kg

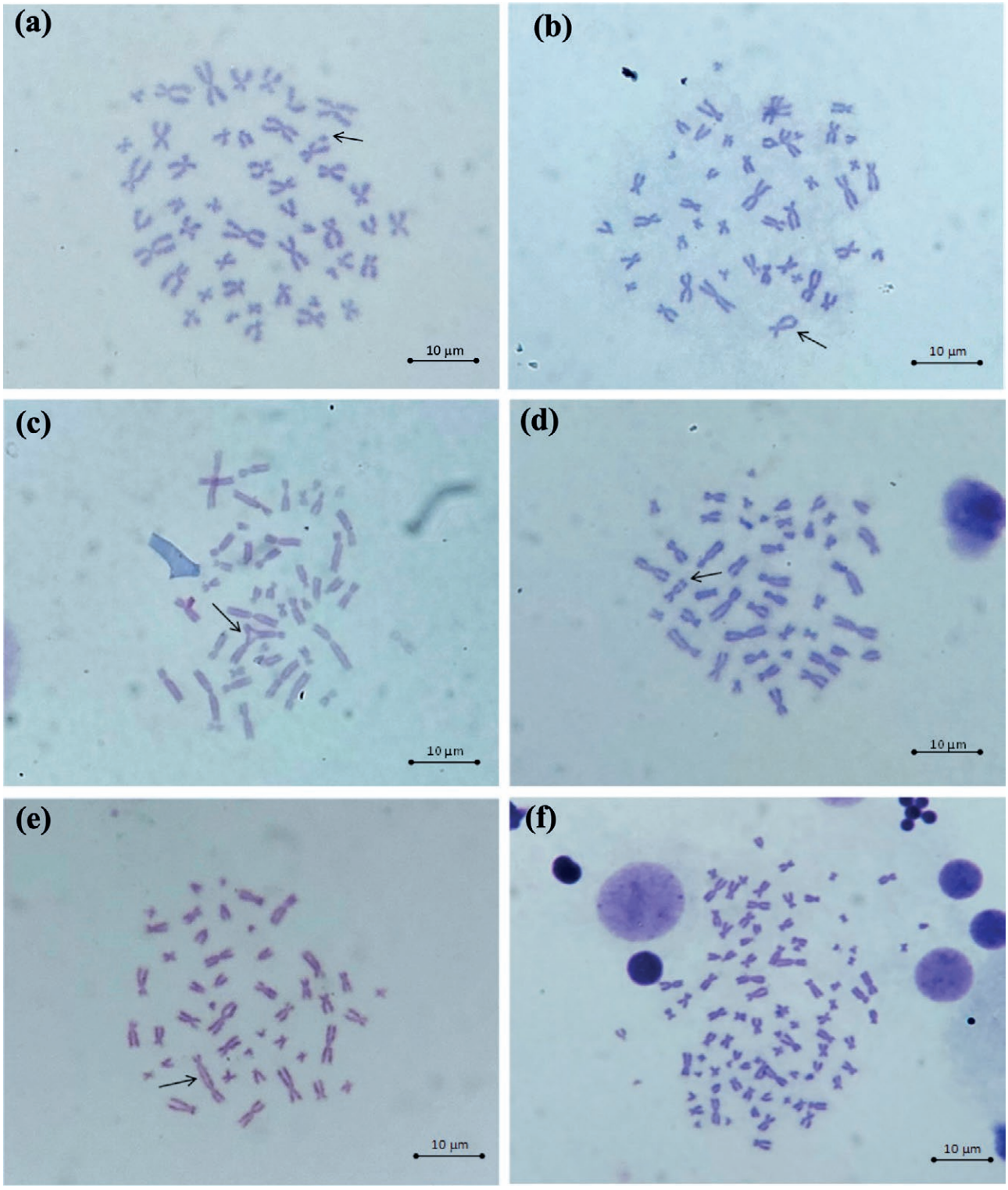

Figure 2. Different types of chromosomal aberrations induced by forchlorfenuron in human lymphocytes (a) chromatid break, (b) sister chromatid union, (c) chromatid exchange, (d) chromosome break, (e) dicentric chromosome., and (f) polyploid cell.

Table 1. Chromosome Aberrations in Human Peripheral Blood Lymphocytes Treated with Different Concentrations of Forchlorfenuron for 24 and 48 h.

Test substance	Treatment		Structural CAs						Numerical CA	Total CA	% cells with structural CAs±SD	Total CAs/ cell±SD
	Time (h)	Conc. (µg/mL)	B'	SU	CE	B"	F	DS	P			
Negative control	-	-	14	9	0	0	0	0	0	23	5.75±0.95	0.058±0.01
Mitomycin-C	24	0.20	53	6	7	14	0	0	0	80	19.50±2.64a_3	0.200±0.02a_3
Forchlorfenuron	24	0.25	20	9	0	0	0	1	1	31	7.25±1.50b_3	0.076±0.01b_3
		0.50	25	10	0	0	0	0	1	36	8.00±1.63b_3	0.090±0.01a_1b_3
		1.00	24	9	1	5	0	0	0	39	9.75±2.36a_1b_3	0.098±0.02a_1b_3
		2.00	25	16	0	2	0	0	1	44	10.25±2.50a_2b_3	0.110±0.05a_2b_3
Mitomycin-C	48	0.20	49	9	12	14	1	1	0	86	20.75±3.86a_3	0.220±0.03a_3
Forchlorfenuron	48	0.25	21	8	0	3	0	0	0	32	7.75±2.87b_3	0.080±0.02b_3
		0.50	24	12	0	0	0	0	1	37	9.00±2.44b_3	0.093±0.02a_1b_3
		1.00	29	11	0	2	0	0	1	43	10.00±2.16a_1b_3	0.108±0.01a_2b_3
		2.00	25	15	1	1	0	0	2	44	10.25±0.95a_1b_3	0.110± 0.01 a_2b_3
#Frequency of aberrations (%):			63.07	29.41	0.65	4.25	–	0.33	2.29			

All data are given as mean±SD; n=4.
In the CA test, 400 cells in total were scored in each concentration.
MMC: Mitomycin; B': Chromatid break; SU: Sister union; CE: Chromatid exchange; B": Chromosome break; F: Fragment; DS: Dicentric chromosome.
a, significant from negative control; b, significant from Mitomycin-C (positive control).
a_1b_1: $P<0.05$; a_2b_2: $P<0.01$; a_3b_3: $P<0.001$.
#: Data from the positive and negative controls were not taken into account when calculating the frequency of aberrations (%).

Table 2. Mitotic Index values in Human Peripheral Blood Lymphocytes Treated with Different Concentrations of Forchlorfenuron for 24 and 48 h.

Test Substance	Treatment		Total Counted Cells	Total Dividing cells	Mitotic Index±SD
	Time (h)	Conc. (µg/ml)			
Negative control	-	-	8000	470	5.88 ± 0.40
Mitomycin-C	24	0.20	8000	281	3.51 ± 0.57 a_3
Forchlorfenuron	24	0.25	8000	464	5.80 ± 0.16 b_3
		0.50	8000	383	4.79 ± 0.59 a_1b_1
		1.00	8000	296	3.70 ± 1.26 a_3
		2.00	8000	305	3.81 ± 0.82 a_3
Mitomycin-C	48	0.20	8000	271	3.39 ± 0.46 a_3
Forchlorfenuron	48	0.25	8000	336	4.20 ± 0.98 a_2
		0.50	8000	276	3.45 ± 0.92 a_3
		1.00	8000	258	3.23 ± 0.66 a_3
		2.00	8000	255	3.19 ± 1.14 a_3

a, significant from negative control; b, significant from Mitomycin-C (positive control).
a_1b_1: $P<0.05$; a_2b_2: $P<0.01$; a_3b_3: $P<0.001$.

b.w for 180 days. The researchers reported severe uterine hydrometra, pathological ovarian alterations, and possible negative effects of FCF on the ovaries and steroidogenesis in rats. Although no other research was found regarding the genotoxic potential of FCF, the genotoxic effects of various synthetic PGRs were described in earlier studies conducted on a variety of test systems, which are consistent with our findings (Kocaman and Bucak 2016; Kocaman and Güven 2016; Özkul et al. 2016; Kocaman and Kılıç 2017; Ozel et al. 2022).

As is well known, while FCF plays a role in cell growth and differentiation by promoting cell division in plants (Vardi-Oknin et al. 2013), it disrupts higher-order septin assembly in other eukaryotic organisms. It was reported that in mammalian cells, FCF specifically and directly changes septin assembly without influencing actin or tubulin polymerization (Hu et al. 2008). At the same time, considering that septin polymers are involved in the organization of various structures such as contractile rings, actin stress fibrils, and mitotic spindle in animals (Kinoshita 2006); in our study, it can be thought that FCF might have inhibited cytokinesis, especially by acting on septins, thus causing the formation of polyploid cells. However, in this study, it should be emphasized that FCF significantly induced structural CAs, especially chromatid breaks and sister chromatid unions indicating that it effectively acts as a clastogenic agent by breaking the phosphodiester backbone of DNA.

In this study, FCF generally showed a cytotoxic effect in human lymphocyte cultures by significantly decreasing the MI compared to the negative control. Zhang et al. (2015) reported that FCF exhibited a cytotoxic effect on Chinese hamster ovary cells, which is in line with the findings of our investigation. Additionally, it was revealed that exposure to FCF derivatives markedly decreased the proliferative capacity of human and mouse malignant mesothelioma cells and led to cytotoxicity (Blum et al. 2019). In recent years, FCF has been employed as an experimental tool for putative therapeutic applications in cancers since it disturbs the higher-order assembly of septins in non-plant eukaryotes (Blum et al. 2019). However, Ivanov et al. (2021) reported that FCF might not be convenient for therapeutic usage in its current form since only active at high concentrations. Animal tissue toxicity and potential off-target effects of FCF have also been documented (Heasley and McMurray 2016; Sun et al., 2020). It was therefore suggested by researchers that caution should be warranted when using FCF to study the biological functions of septins in cellular systems and model organisms (Sun et al. 2020).

The cytotoxic potential of FCF that was observed in this investigation may be due to its disrupting the septin organization in human lymphocyte cells. Hu et al. (2008) reported that FCF suppresses normal septin dynamics in mammalian cells and stabilizes septin polymers, causing alterations in cell shape, mitotic abnormalities, and a reduction in cell motility. Additionally, septins have been associated with the regulation of the cell cycle (Barral et al. 1999; Shulewitz et al. 1999), coordination of the response to DNA damage, and cellular shape (Lew 2003; Keaton and Lew 2006; Enserink et al. 2006; Smolka et al. 2006; Weirich et al. 2008). Blum et al. (2019) reported that the chloride group in the structure of FCF has an important role in binding to septins with a high affinity and thereby inhibiting cell proliferation. It can be thought that the formation of thick septin bundles by stabilizing the septins with the effect of FCF may lead the cell to apoptosis by disrupting the signal transmission traffic.

In conclusion, this study indicated for the first time that commercial formulation of FCF could have genotoxic and cytotoxic effects on human lymphocytes at concentrations ranging from 0.50 to 2.00 µg/ml. It is clear that ensuring the use of PGRs, containing FCF as an active ingredient, in appropriate concentrations in agricultural applications can reduce the risk of this probable toxic substance being released into the environment and the negative effects on human health through residues in vegetables and fruits. It should also be noted that it is important for people exposed to FCF during production or application to wear personal protective gear such as gloves, masks, face shields, and goggles. Finally, it may be said that this study is important in that it encourages further *in vivo* and *in vitro* genotoxicity studies by drawing attention to this widely used PGR.

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