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Assessment of DNA Damage Induced by Velum® Prime in Human Lymphocytes

Velum® Prime Kaynaklı DNA Hasarının İnsan Lenfositlerinde Değerlendirilmesi

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Abstract

Aim: Fluopyram is a mitochondrial complex II inhibitor with low water solubility and a relatively long half-life in soil. So it may also be dangerous for humans. It is very likely to reach humans with its widespread use and long-term stay in nature. Therefore, its genotoxicity should be fully demonstrated.

Material and Method: The effect of fluopyram on DNA damage was evaluated in human lymphocytes using the comet assay. Lymphocytes of eight volunteers were isolated using histopaque-1077. Fluopyram was administered at doses of 0.05, 0.25, and 1.00 mg/mL for 1, 2, and 4 h. The comet assay was applied, and photographs of the slides were taken under a fluorescence microscope. 50 cells per slide were analyzed using the OpenComet software. The obtained results were statistically evaluated using one-way ANOVA.

Results: Fluopyram treatments at 1.00 mg/mL for 1 h and 0.05, 0.25, and 1.00 mg/mL for 2 and 4 h resulted in a statistically significant increase in DNA damage compared to the internal control groups ($p<0.05$). When comparing groups with the same treatment time but different doses, the increase in DNA damage observed after a 1-h treatment of 1.00 mg/mL fluopyram was higher than the increase observed after a 1-h treatment of 0.05 mg/mL fluopyram ($p<0.05$). When comparing groups with different treatment times but the same dose, the increase in DNA damage after a 4-h treatment of 0.25 mg/mL fluopyram was higher than the increase observed after a 1-h treatment of 0.25 mg/mL fluopyram ($p<0.05$).

Conclusion: The results suggest that fluopyram causes an increase in DNA damage in a dose- and time-dependent manner. It is essential to investigate these findings in vivo as well.

Keywords: Comet assay, fluopyram, genotoxicity, pesticide

Öz

Amaç: Fluopyram mitokondriyal kompleks II inhibitörü, suda çözünürlüğü düşük ve topraktaki yarılanma ömrü oldukça uzun bir pestisittir. Yaygın kullanımı ve doğada uzun süreli kalabilmesi ile insanlara ulaşması oldukça muhtemeldir. Dolayısı ile genotoksisite riski tam olarak ortaya konmalıdır.

Gereç ve Yöntem: Fluopyramın DNA hasarı üzerindeki etkisi insan lenfosit hücrelerinde comet metodu ile değerlendirilmiştir. 8 gönüllüden histopak-1077 kullanılarak lenfositler elde edilmiştir. 0,05, 0,25 ve 1,00 mg/mL olmak üzere 3 dozda ve 1, 2 ve 4 saat fluopyram uygulaması yapılmıştır. Comet metodu uygulanmış ve hazırlanan preperatların floresan mikroskop altında fotoğrafları çekilmiştir. Preperat başına 50 hücre OpenComet programı ile değerlendirilmiş ve sonuçlar tek yönlü anova ile istatistiksel olarak değerlendirilmiştir.

Bulgular: 1,00 mg/mL 1 saat ve 0,05, 0,25, 1,00 mg/mL 2 ve 4 saat fluopyram uygulamaları internal kontrol gruplarına kıyasla DNA hasarında istatiksel olarak anlamlı artışa sebep olmuştur ($p<0,05$). Aynı uygulama süresine ve farklı doza sahip gruplar kendi arasında karşılaştırıldığında, 1 saat 1,00 mg/mL fluopyram uygulaması sonucunda DNA hasarında meydana gelen artış, 1 saat 0,05 mg/mL fluopyram uygulaması sonucunda meydana gelen artıştan daha yüksektir ($p<0,05$). Farklı uygulama süresine ve aynı doza sahip gruplar kendi arasında karşılaştırıldığında 4 saat 0,25 mg/mL fluopyram uygulaması sonucunda DNA hasarında meydana gelen artış, 1 saat 0,25 mg/mL fluopyram uygulaması sonucunda meydana gelen artıştan daha yüksektir ($p<0,05$).

Sonuç: Bu sonuçlara göre fluopyramın doz ve zaman bağımlı şekilde DNA hasarında artışa sebep olduğu tespit edilmiştir. Sonuçların in vivo olarak da araştırılması gerekmektedir.

Anahtar Kelimeler: Comet metodu, fluopyram, genotoksisite, pestisit

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INTRODUCTION

DNA damage is a significant concern that can play a role in the development of cancer and many other chronic diseases.[1] Furthermore, it also plays a role in certain vital intracellular physiological events, such as p53-mediated apoptosis.[2] Therefore, understanding potential DNA damage is crucial for preventing various problems. DNA damage can be caused by various endogenous or exogenous reasons, with chemicals being one of these exogenous sources.[3] Pesticides, which we frequently encounter in our daily lives, are among the harmful chemicals and may cause DNA damage.[4-9] While pesticides are essential for efficient agricultural production, they have been implicated as possible factors behind the rising incidence of certain diseases.[10] With the rapid increase in pesticide use, health issues have also escalated.[11] Pesticide production is a dynamic process, and new formulations or pesticides are continuously introduced to the market. Each pesticide must be investigated individually, and its potential harm to human health must be identified. This includes the adverse effects they might cause on DNA.

Fluopyram, (FL, 396.72 g/mol, C16H11ClF6N2O, CAS Number: 658066-35-4), initially developed by Bayer as a fungicide[12] is a relatively new pesticide currently employed as a nematicide.[13] It comes in various formulations containing different amounts of the active ingredient and is also available in combined formulations with other pesticides. It gained widespread use due to its lack of cross-resistance with previous fungicide families[14] FL functions by inhibiting succinate dehydrogenase (SDH, Complex II) in the mitochondrial respiratory chain, making it a member of the succinate-dehydrogenase inhibitors (SDHI) class of fungicides.[12] SDH is composed of four protein subunits (SDHA-D). The succinate binding region resides within SDHA, and the Ubiquinone (coenzyme Q) binding site formed by the other subunits is blocked by FL.[15] Inhibition of succinate dehydrogenase halts ATP production, ultimately leading to cell death.[15]

The water solubility of FL is low, and its half-life in soil reaches up to two years, which is relatively longer compared to similar pesticides.[16] Therefore, it is highly likely to affect humans after application. However, studies on the genotoxicity of FL are almost non-existent, and there has been no investigation conducted to evaluate DNA damage in human lymphocytes. In conclusion, the objective of this study is to examine the impact of FL on DNA damage, considering its widespread use and potential for long-term environmental persistence, which makes it highly likely to affect humans. For this purpose, the effects of Velum® Prime, a product exclusively containing FL as the active ingredient and manufactured by Bayer, on DNA were investigated in vitro using the comet assay[17] a method capable of rapidly and accurately measuring DNA damage.

MATERIAL AND METHOD

The study was carried out with the permission of Süleyman Demirel University Faculty of Medicine Clinical Researches Ethics Committee (Date: 10.10.2022, Decision No: 285). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Study Design

The volunteers included in the study were selected based on various exclusion criteria. Accordingly, the study included four female and four male volunteers aged between 18 and 45, who had no chronic illnesses or continuous medication use, had not undergone any examination or radiation treatment in the last six months, and were non-smokers. The study was conducted in accordance with the principles of the "Helsinki Declaration," and informed consent was obtained from the volunteers. A total of 15 mL of blood was collected from each volunteer, and the blood samples from all volunteers were utilized separately for 15 different groups. The groups are presented in **Table 1**. The doses applied were selected based on the findings of previous studies.[18] The Velum® Prime (Bayer AG, Suspension concentrate, 400 g/l FL) used in the research was obtained from local vendors. All chemicals mentioned as used in the study were obtained through local vendors from Sigma (St. Louis, MO, US) or Merck (Darmstadt, Germany). The manufacturers of chemicals not obtained from these companies are given in parentheses.

Table 1. Groups and doses in the study

Groups	n	Duration	Application
1	8	1 h	None
2			0.05 mg/mL FL
3			0.25 mg/mL FL
4			1.00 mg/mL FL
5			100 µM H_2O_2
6	8	2 h	None
7			0.05 mg/mL FL
8			0.25 mg/mL FL
9			1.00 mg/mL FL
10			100 µM H_2O_2
11	8	4 h	None
12			0.05 mg/mL FL
13			0.25 mg/mL FL
14			1.00 mg/mL FL
15			100 µM H_2O_2

Comet Assay

The assay was performed in accordance with the "OECD In Vivo Mammalian Alkaline Comet Assay Guideline".[19] Blood was drawn from the volunteers, and the comet assay procedure was initiated immediately. Blood samples were mixed in a 1:1 ratio with Histopaque-1077 and centrifuged at 2000 RPM for 20 min, allowing the separation of lymphocytes. These lymphocytes were mixed in a separate tube at a 1:1 ratio with PBS and then centrifuged at 2500 RPM for 10 min. Subsequently, the PBS was

removed, and the lymphocytes were supplemented with RPMI 1640 containing 10% FBS, adjusting the final volume to 1 mL before proceeding to the FL treatment. In accordance with the doses specified in **Table 1**, the FL treatment was conducted at three different time intervals (1, 2, and 4 h) in an incubator at 37°C. For each time interval, separate internal negative and positive control groups were established. 100 µM H_2O_2 was used as the positive control. Following the incubations, the cells were centrifuged at 2500 RPM for 10 min to separate and then washed with PBS and centrifuged again at 2500 RPM for another 10 min. Subsequently, all groups were incubated for an additional 1 h at 37°C in an incubator. For the detection of DNA damage, 20 µL of cells were mixed with 100 µL of low melting point agarose (0.7%, Fisher Scientific, Massachusetts, USA) and spread onto slides pre-coated with normal melting point agarose (1%, Serva Electrophoresis, Germany). The slides were incubated in cold lysis solution (pH: 10, 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100) in the dark at +4°C for 90 min. Following this procedure, samples were incubated in ice-cold electrophoresis solution (pH: 13, 300 M NaOH, 1 mM EDTA) in the dark at +4°C for 30 min. Subsequently, an electrophoresis procedure was carried out at 25 V (1.02 V/cm) and +4°C for 25 min. After the designated time, the slides were carefully removed from the electrophoresis tank, rinsed three times with neutralization solution, and then left to dry. During the imaging phase, the slides were stained with 20 µL of fluorescent dye (ethidium bromide) and examined under a microscope (Zeiss Imager A1 fluorescence microscope). Two preparations were prepared from each sample and photographs of 50 cells per slide were taken randomly with a camera (Axiocam Icc 1). The photographs were analyzed using the OpenComet software.[20] The Tail DNA Percentage (TDNAP) parameter was used as an indicator of DNA damage.

Statistical Analysis

The obtained results were statistically evaluated using one-way ANOVA (posthoc Tukey) in SPSS v29.[21] software. Results are presented as mean±standard error, and a p-value of <0.05 was considered statistically significant.

RESULTS

Groups treated with different doses of FL and 100 µM H_2O_2 (positive control) have shown an increase in DNA damage compared to internal negative control groups. When statistically compared, significant results were obtained for some groups (**Figure 1**). Despite more DNA damage was detected compared to the negative control groups, the DNA damage observed in the FL-treated groups is significantly lower than the DNA damage observed in the internal positive control groups.

As expected, the positive control groups caused significantly higher levels of DNA damage compared to all groups with the same time interval ($p<0.05$).

When the groups 2, 3, and 4, subjected to 1-h/0.05-0.25-1.00 mg/mL FL treatments respectively, are compared to internal negative control group an increase in DNA damage is observed. However, this increase in groups 2 and 3; is not statistically significant ($p>0.05$). The increase in DNA damage observed in group 4, on the other hand, is statistically significant ($p<0.05$). When groups 2, 3, and 4 are compared among themselves, it is determined that the group subjected to 1-h/1.00 mg/mL FL treatment causes statistically significantly more DNA damage than the group subjected to 1-h/0.05 mg/mL FL treatment ($p<0.05$). Accordingly, a dose-dependent increase in DNA damage is observed in the 1-h FL treatments.

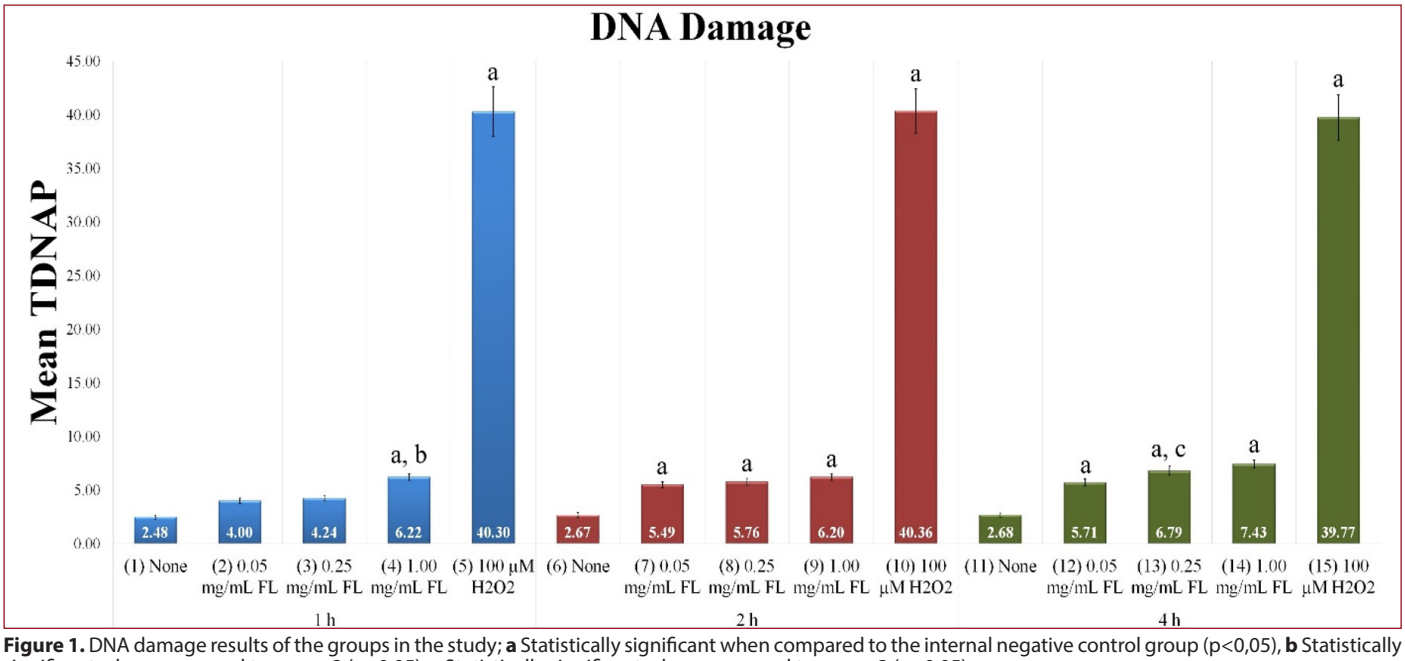

Figure 1. DNA damage results of the groups in the study; **a** Statistically significant when compared to the internal negative control group ($p<0,05$), **b** Statistically significant when compared to group 2 ($p<0,05$), **c** Statistically significant when compared to group 3 ($p<0,05$)

When the groups 7, 8, and 9, subjected to 2-h/0.05-0.25-1.00 mg/mL FL treatments respectively, compared to internal negative control group, an increase in DNA damage is observed for all three groups ($p<0.05$). When groups 7, 8, and 9 are compared among themselves, it is observed that DNA damage increases as the dose increases; however, this increase is not statistically significant ($p>0.05$). Accordingly, a dose-independent increase in DNA damage has been observed in the 2-h FL treatments.

When the groups 12, 13, and 14, subjected to 4-h FL/0.05-0.25-1.00 mg/mL treatments respectively, compared to internal negative control group, an increase in DNA damage is observed for all three groups ($p<0.05$). When groups 12, 13, and 14 are compared among themselves, it is observed that DNA damage increases as the dose increases; however, this increase is not statistically significant ($p>0.05$). Accordingly, a dose-independent increase in DNA damage has been observed in the 4-h FL treatments.

Groups with varying time intervals but identical FL doses were also compared among themselves. When compared the groups 2, 7, and 12, which subjected to 0.05 mg/mL/1, 2 or 4-h FL treatment respectively, it was observed that as the time increased, DNA damage increased; however, this increase was not statistically significant ($p>0.05$). When compared the groups 3, 8, and 13, which subjected to 0.25 mg/mL FL/1, 2 or 4-h treatment respectively, it was observed that as the time increased, DNA damage increased. When comparing the 1-h and 2-h treatments, no significant difference was observed ($p>0.05$). Similarly, when comparing the 2-h and 4-h treatments, no significant difference was observed ($p>0.05$). However, when comparing 4-h/0.25 mg/mL treatment with 1-h/0.25 mg/mL treatment, it was determined that the increase in DNA damage was statistically higher ($p<0.05$). Moreover, when compared the groups 4, 9, and 14, which subjected to 1.00 mg/mL/1, 2 or 4-h FL treatment respectively, it was observed that as the time increased (4 h), DNA damage increased; however, this increase was not statistically significant ($p>0.05$).

According to these results, it can be concluded that FL causes an increase in DNA damage in a dose and time-dependent manner.

DISCUSSION

Among the over 200 fungicides listed by the Fungicide Resistance Action Committee, succinate dehydrogenase inhibitors constitute the most rapidly expanding class in terms of newly synthesized and introduced compounds,[22] with FL being one of the most extensively employed active substances among these pesticides owing to its utilization as a nematicide. Nevertheless, the number of studies addressing the health impacts of FL is quite limited. Apart from Complex II inhibition, its mode of action, side effects in various organisms, and cumulative effects remain uncertain. Its widespread use leads to contamination in both soil and water. In a study conducted in Denmark, the presence of chemicals such as pesticides and pharmaceuticals was investigated in various freshwater systems, and among 83 chemicals examined, FL emerged as one of the most prevalent and widespread substances.[23] In a study published in 2023 and conducted in Austria, the feed of dairy cattle was analyzed for over 700 pesticides and pharmaceuticals, resulting in the identification of a total of 16 compounds. Among these compounds, FL emerged as the most prevalent, accounting for 62% of the total findings. Moreover, FL has been identified as the pesticide that most frequently exceeds the Maximum Residue Level (MRL) limits set by the European Union. In the study, it was found that the widespread presence of pesticides at low doses in the food/feed chain could have implications for animal, human, and environmental health.[24] In light of these results, there have been emerging concerns about the ecotoxicological implications of FL.[18]

Besides its SDH inhibition, it has been determined that FL also induces oxidative stress in nematodes and leads to an increase in reactive oxygen species (ROS).[18] As is well-known, one of the primary causes of DNA damage is oxidative stress.[25] In a study conducted with Luna® Experience, which contains 200 g/L FL and 200 g/L tebuconazole as active ingredients, rats were administered pesticide doses of 5, 10, and 20 mg/kg. Subsequently, oxidative stress markers in the liver and blood, as well as DNA damage, were examined. Both in the blood and the liver, a decrease in catalase enzyme activity and an increase in DNA damage were observed. It was concluded that this DNA damage arose from oxidative stress.[26] In another study published by the same team, using the same experimental design, the cytotoxicity and genotoxicity of Luna® Experience at doses of 5, 10, and 20 mg/kg were evaluated in rat bone marrow. The pesticide demonstrated both cytotoxic and genotoxic properties across all administered doses.[27] In our study, FL was identified as genotoxic, and existing research, albeit in its preliminary stages, suggests that this toxicity may be attributed to oxidative stress. In another study, the effect of FL on tumor formation in the liver was investigated. In female rats exposed to FL for 3, 7, or 28 days at doses of 30, 75, 150, 600, or 1500 ppm, hepatocellular adenoma and carcinoma formation mediated by constitutive androstane receptor/pregnane X receptor activation was observed at the 1500 ppm dose. In the study, the pathway involving DNA damage, which contributes to the formation of liver tumors, was not investigated, given that FL had not been previously reported as genotoxic. FL has been identified as a potential carcinogen for liver tumors.[12]

In addition to these limited studies, there are also researches conducted with pesticides belonging to the same class as FL (complex II inhibitors). The genotoxic and cytotoxic effects of the fungicide Signum and its active constituents (boscalid and pyraclostrobin) on human peripheral blood lymphocytes were investigated using the micronucleus test. The investigation included the evaluation of micronuclei, nucleoplasmic bridges, nuclear bud formations, and

the cytokinesis-block proliferation index. Micronucleus formation statistically increased at doses of 0.5 and 2 µg/mL boscalid, 0.5, 1.5, and 2 µg/mL pyraclostrobin, and 2, 6, and 25 µg/mL signum, while nucleoplasmic bridges increased at a dose of 0.25 µg/mL pyraclostrobin. Although there is no statistically significant increase in nuclear budding formation, it has been determined that cytotoxicity rises in correlation with concentration. It has been concluded that Signum, boscalid, and pyraclostrobin may exhibit genotoxic and cytotoxic effects in lymphocytes.[28] In a study evaluating bixafen, similar to FL, it was determined that bixafen is genotoxic at low doses in the human neuroblastoma cell line (SH-SY5Y) and T-cell leukemia cell line (Jurkat), and it has been suggested that the mechanism could be oxidative stress-induced DNA damage due to increased ROS activity.[29] Benzovindiflupyr also operates through the SDHI mechanism. The toxicity on earthworms (*Eisenia fetida*) has been assessed at doses of 0.1, 1, 5, and 10 mg/kg. It has been found that at high doses, it significantly inhibits mitochondrial complex II and concurrently leads to a substantial increase in ROS and lipid peroxidation. It has also been observed that it causes an increase in DNA damage in a dose and time-dependent manner.[30] The potential cytotoxic/genotoxic effects of another SDH inhibitor, benodanil, were evaluated in onion root meristem cells using the mitotic index and in vitro human peripheral blood lymphocytes using the micronucleus test. At concentrations of 12.5, 25, and 50 ppm, the mitotic index and prophase index decreased compared to the control group in the presence of benodanil. Besides, benodanil significantly reduced the nuclear division index.[31] In a study investigating the toxic effects of the SDH inhibitor penthiopyrad on zebrafish, it was determined that there is an increase in oxidative stress in the liver tissue. Additionally, disruptions were observed in mitochondrial respiratory complexes, mtDNA synthesis, lipid metabolism, and alterations were detected in the expression of genes associated with apoptosis. It was concluded that penthiopyrad toxicity leads to disruptions in lipid metabolism, mitochondrial dysfunction, apoptosis, and DNA damage.[32] In another study conducted on zebrafish, the fish were exposed to 0.25, 50, and 1000 µg/L of flutolanil for 60 days. The research findings reveal that there is a noteworthy reduction in catalase activity in the liver across all groups, coupled with an elevation in malondialdehyde levels, and a dose-dependent increase in DNA damage has been observed as well. Following chronic exposure to flutolanil, alterations in the transcription levels of genes involved in apoptosis and the immune system have been reported, along with an increase in caspase-3 enzyme activity.[33]

According to European Food Safety Authority (EFSA) 2023 report about permitted maximum residue levels (MRL) of FL in different fruits and vegetables, recommended MRL levels of FL range from 0.01 to 40 mg/kg.[34] In a study published in 2023, the FL residues in different fruits and vegetables was investigated. Except for the high value in one tomato sample, all residue amounts were detected in accordance with EFSA MRL levels.[35] In our study, doses between these ranges were used and the genotoxicity of FL was evaluated. Although doses were administered directly to lymphocytes, it could mean that even permitted/suggested MRL doses of FL in the long term are likely to be genotoxic.

Considering the studies indicating the potential genotoxicity of other pesticides acting as SDH inhibitors, it is evident that further comprehensive investigations are necessary for FL, a member of the SDHI group. The findings of our study can serve as a precursor to more advanced mechanistic research; however, it is important to acknowledge certain limitations. One of these limitations is the fact that our study was conducted within a lymphocyte culture. Hence, it has not been possible to determine the specific impact of FL within a metabolism on DNA damage. Secondly, our research evaluated short-term exposures. However, the cumulative effects that may arise from longer, sub-chronic, or chronic exposures should also be taken into account and evaluated.

CONCLUSION

With respect to the results of our study, FL leads to an increase in DNA damage in a dose and time-dependent manner. Even after short-term and low-dose exposures, there has been an increase in DNA damage, although not always statistically significant. The increase in DNA damage becomes more significant with higher doses or longer exposure times. The DNA damage observed may not necessarily result in diseases or cell death and could be effectively repaired by DNA repair mechanisms. Therefore, before arriving at a definitive judgment that FL is unequivocally genotoxic, more comprehensive in vitro and in vivo studies are needed. Nonetheless, results obtained from the lymphocyte culture medium indicate that FL might pose a risk in terms of DNA damage.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Süleyman Demirel University Faculty of Medicine Clinical Researches Ethics Committee (Date: 10.10.2022, Decision No: 285).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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