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RESECRH ARTICLE

FENTANYL GENOTOXICITY EVALUATION VIA COMET ASSAY

Öge ARTAGAN [1,*], Bahar KÖKLÜ [2]

[1] Medical Services and Techniques, Vocational School of Health Services, Muğla Sıtkı Koçman University, Muğla, Turkey
ogebasoglan@mu.edu.tr - 0000-0001-9389-4450

[2] Biology, Instution of Graduation Schools, Eskişehir Technical University, Eskişehir, Turkey
baharr263@gmail.com - 0009-0007-0316-6769

Abstract

DNA is our main genetic structure that can be affected by various molecules we come into contact with. Therefore, there is a high probability of DNA damage. Genotoxicity tests involve systematic methods designed to assess the safety of drugs and chemicals. Comet assay evaluates the possible genotoxicity resulting from DNA single-strand breaks.

Fentanyl is a powerful painkiller from the group called opioid analgesics administered intravenously. In this research, we evaluated the possible DNA damage in human lymphocytes after fentanyl exposure via single-cell gel electrophoresis (Comet Assay).

DNA damage in healthy human peripheral lymphocytes treated with fentanyl was investigated via comet assay. Lymphocytes were treated with 5, 10, 20, and 40 µg/mL doses of fentanyl for 1 hour. After the incubation period, the cells' DNA tail length, tail intensity, and tail moment values were evaluated by comparing them with the spontaneous control and positive control data. EtBr stained slides were visualized under the fluorescent microscope. Fentanyl induced the comet parameters such as tail length, tail intensity, and tail moment but dose-dependent increase was not obtained after fentanyl administration. Fentanyl showed the highest tail length and tail moment value at the dose of 10 µg/mL. The highest tail intensity value was obtained at the dose of 40 µg/mL fentanyl administration.

This study aimed to reveal previously undiscovered genotoxicity of fentanyl on healthy human lymphocytes in vitro via comet assay. Fentanyl exposure induces DNA damage in healthy human lymphocytes, as shown via comet parameters (tail length, tail intensity, and tail moment). Genotoxic effect does not display a consistent dose-dependent increase.

Keywords

Comet assay,
Single-cell gel electrophoresis,
DNA damage,
Lymphocyte culture,
Genotoxicity,
Fentanyl

Time Scale of Article

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1. INTRODUCTION

Genotoxic effects of anesthetic agents are very important research area [1,2]. Applications for determining genotoxic damage in patients due to frequent exposure to anesthetics are important. Further studies with comet assay is necessary to clarify and follow up the extent of genotoxic damage.

In this research, possible genotoxic effects of fentanyl was evaluated in human lymphocytes via comet assay. Fentanyl is a powerful rapid-acting painkiller, originally developed in 1960 by Dr. Paul Janssen [3]. Because of its potency, effectiveness, and rapid onset of action, Fentanyl is one of the most widely

*Corresponding Author: ogebasoglan@mu.edu.tr

used opioids and is commonly used to manage acute and chronic pain. Fentanyl is approximately 50-100 times more potent than the known natural opioid morphine [4,5].

Lymphocytes were chosen for the comet assay due to their high sensitivity to DNA damage, reflecting systemic exposure to genotoxic agents. Their non-invasive sampling makes them ideal for assessing whole-body effects. Given fentanyl's impact on multiple organ systems, evaluating its genotoxicity in lymphocytes provides insight into potential systemic toxicity [6].

Comet assay has been shown to be a highly sensitive, rapid, visual and rational method for the evaluation of DNA single strand breaks in individual cells [7, 8]. The effects of opioids, such as fentanyl, on immune function are a critical issue that highlights the importance of dosage. In a clinical study, the doses of fentanyl administered to healthy individuals were examined. Participants received an initial dose of fentanyl of 3 micrograms/kg via IV, followed by a 2-hour IV infusion at a dose of 1.2 micrograms x kg (-1) x h (-1). Various tests were conducted to evaluate immune function before and after fentanyl administration. The analysis revealed that fentanyl did not suppress immune resistance; in fact, it increased natural killer cell cytotoxicity. Results suggest that careful consideration of dosage is essential for understanding the effects of opioids, such as fentanyl, on immune system [9].

It is very important to determine DNA damage in patients, especially in repeated anesthesia applications, and it is very important to reveal genotoxicity with the comet assay to clarify the degree of damage that may occur in case of frequent exposure to anesthetics such as fentanyl [10, 7].

Researches on genotoxic of fentanyl is limited. Genotoxicity of fentanyl has been investigated and results were reported with bacterial mutation assay [11]. To our knowledge, no such studies have been conducted with fentanyl in vitro comet assay by using human lymphocytes [12].

As understood from these information, genotoxicity of fentanyl data was limited so 5, 10, 20, and 40 µg/mLof fentanyl concentrations were applied on human lymphocyte cultures in vitro.

2. METHODS

The aim of the comet assay is to achieve at least 80% viability in cells exposed to an agent. Based on this result, fentanyl concentrations of 5, 10, 20, and 40 µg/mL were used for the comet experiments.

The peripheral blood samples were then transferred to tubes containing Histopaque and centrifuged at room temperature and centrifuged 400 rcf for 30 minutes. Lymphocytes separated due to the density difference after centrifugation are added to the lymphocytes in these falcons with 6 mL of PBS solution washed with PBS solution. The separated lymphocytes were transferred to separate eppendorf tubes and Trypan blue staining was used to confirm viability above 80% in isolated lymphocytes. The samples were subsequently placed in remifentanil concentrations and incubated for an hour at 37°C. At the end of the incubation period, eppendorf tubes were centrifuged at 300 rpm for 5 minutes, the supernatant was removed. Next, 100 µL of a low-melting-point agar, mixed with the 100 µL lymphocyte solution, was dripped onto slides previously coated with high-melting-point agar. These slides were then covered with a 24 x 60 mm coverslip. After incubating the slides in the refrigerator for 15-20 minutes, the coverslips were separated from the slides and placed in chalets containing a lysis solution. They were kept in the refrigerator for 1 hour. Following lysis, the slides were placed in horizontal tanks containing an electrophoresis buffer solution with a pH greater than 13 for 20 minutes to denature the DNA. Electrophoresis was conducted at 25 V and 300 mA in running buffer [13]. Following electrophoresis, the slides neutralized with a neutralizing buffer (pH 7.5) for 5 minutes [14]. Subsequently, 20 µL of a 20 µg/mL ethidium bromide (EtBr) solution was evenly applied over the slides, which were then covered with a 24 x 60 mm coverslip. All procedures were performed in a darkened area to prevent DNA damage. For comet analysis, the slides were examined at 40x magnification using a fluorescent microscope. Image analysis and comet counting were conducted on 100 comets [15]. Fluorescent EtBr staining was

utilized to detect damage, and images acquired under a fluorescent microscope were analyzed using the Comet Image Processing and Analysis System Software (Comet IV).

2.1. Statistical Analysis

The comet parameters of fentanyl was investigated in 100 cells for each dose of fentanyl, and then statistical analysis was performed by comparing them with the positive and negative control data. Structural changes in tail lengths, tail moments and tail densities were assessed by comparing the number of comets in the cells with the negative and positive controls. These parameters entered into Graphpad Prism 8 program and analyzed separately. Since the variance values between groups in the parameters were different, the Dunnet T3 test was chosen and comparisons were made with the control groups.

3. RESULTS

The clinical relevance of fentanyl's genotoxicity related to its potential to cause genetic damage, which could lead to mutations, cancer, or other genetic disorders. Understanding this aspect is crucial for assessing the long-term risks associated with fentanyl exposure, especially given its widespread medical use and potential for abuse. Currently, direct studies on fentanyl's genotoxic effects are limited. However, research on certain fentanyl analogues provides some insights. A study titled "The Genotoxicity of Acrylfentanyl, Ocfentanyl and Furanylfentanyl" assessed the genotoxic potential of these compounds. The findings indicated that these analogues could induce genetic damage, suggesting a possible genotoxic risk [5]. The aim of the current research is to investigate the potential genotoxicity of fentanyl using the via in vitro comet assay in cultured healthy human lymphocytes, which is worldwide well known genotoxicity test [16, 17, 18, 19, 20].

DNA damage in healhty human peripheral lymphocytes treated with fentanyl was investigated via comet assay. Doses within the range of IC50 or LD50 dose values were used in the comet assay. Lymphocytes treated with 5, 10, 20, and 40 µg/mL doses of fentanyl for 1 hour. After the incubation period the DNA tail length, tail intensity, and tail moment values of the cells were evaluated by comparing them with the spontaneous control and positive control data. EtBr staining was performed and slides were visualised under flourescent microscobe (Figure 1). Fentanyl induced the comet parameters as shown in Figure 2, 3, 4. Fentanyl showed the highest tail length and tail moment value at the dose of 10 µg/mL (Figure 2, 4). The highest tail intensity value was obtained at the dose of 40 µg/mL fentanyl administration (Figure 3).

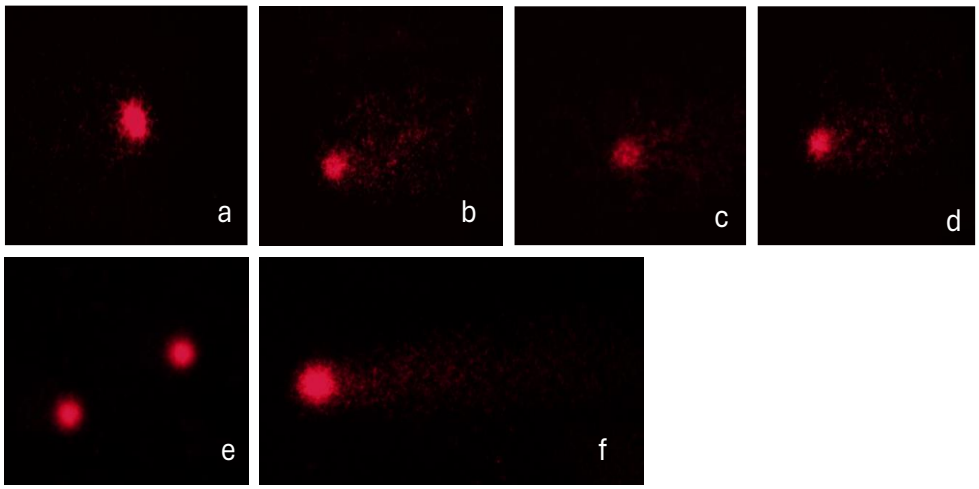

Figure 1. Human peripheral lymphocyte cells treated for 1h with (a) 5 µg/mL (b) 10 µg/mL, (c) 20 µg/mL, (d) 40 µg/mL dose of fentanyl (under 40X) (e) The negative control exhibits intact nuclei with minimal DNA damage, while the positive control which is (f) 50 µM H_2O_2 displays significant DNA fragmentation, characteristic of extensive DNA damage.

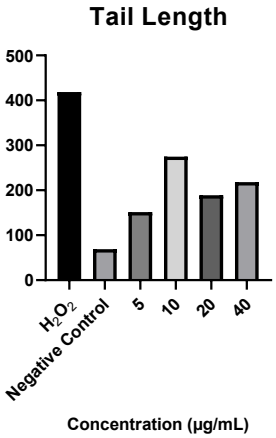

Figure 2. Tail length of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. There is a dose-dependent increase in tail length from 5 to 10 µg/mL. H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for an untreated control.

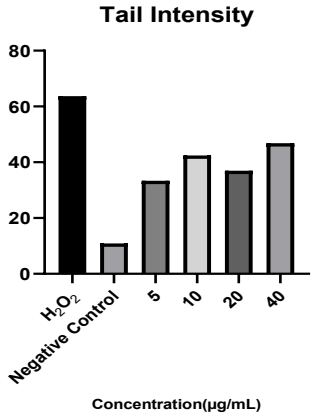

Figure 3. Tail intensity of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. Dose-dependent increase in tail intensity (except 20µg/ml). H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for untreated control.

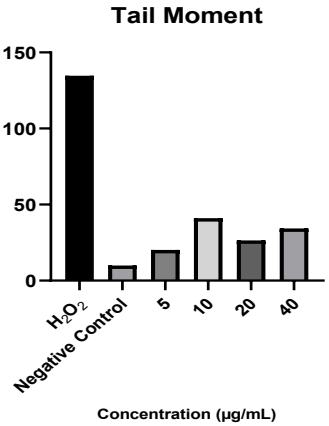

Figure 4. Tail moment of DNA in peripheral lymphocyte cells treated for 1h with doses of fentanyl. H2O2 as a Positive Control, the highest tail length (~450 µm), indicating significant DNA damage. Negative Control, minimal tail length, as expected for untreated control.

Table 1. DNA damage in peripheral lymphocyte cells treated with doses of fentanyl for 1 hour.

	Treatment	**Tail Length**	**Tail Intensity**	**Tail Moment**
	Concentraion (µg/mL)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Negative Control	-	69,00±4,629	10,93±2,221	9,91±4,177
H_2O_2(Positive Control)	50 µM	414,9±12,71***	63,71±4,864***	134,7±11,49***
Fentanyl	5 µg/mL	151,6±63,54***	33,35±23,74***	20,23±18,42**
	10 µg/mL	274,7±119,5***	42,45±22,85***	41,12±31,07***
	20 µg/mL	189,1±99,21	36,96±26,65	26,42±22,98
	40 µg/mL	218,0±71,30***	46,83±25,87***	34,27±27,14***

(Table 1 descriptions) H_2O_2: Hydrogen Peroxide (Positive Control), ±SD: Standard Deviation Values, (Dunnet T3Test), ** P≤ 0.05 *** P≤ 0.001 gives significance values relative to control groups.

Table 1 presents the mean ± standard deviation (SD) of tail length, tail intensity, and tail moment in the comet assay for different treatment groups. The negative control represents untreated cells, while H_2O_2 (50 µM) serves as the positive control to induce significant DNA damage. The statistical significance of differences between treated groups and the negative control was analyzed using Dunnett's T3 test. H2O2: Hydrogen Peroxide (Positive Control), ±SD: Standard Deviation Values, (Dunnet T3Test), ** P≤ 0.05 *** P≤ 0.001 gives significance values relative to control groups.

The comet parameters for fentanyl was investigated in 100 cells for each dose of fentanyl, and then statistical analyze was performed by comparing them with the positive and negative control data. Negative control indicates minimal DNA damage. The positive control (H_2O_2) shows significantly higher comet parameters compared to the negative control. This is consistent with DNA damage caused by oxidative stress and confirms the sensitivity of the test. At the dose of 5 µg/mL, DNA damage increases compared to the negative control, as shown by the comet parameters. Statistical significance indicates that this concentration (10 µg/mL) has a genotoxic effect. DNA damage becomes more evident at the dose of 10 µg/mL, with significant increases in all comet parameters compared to the negative control. This suggests a dose-dependent response. Values of 20 µg/mL dose are slightly lower than 10 µg/mL, although still high compared to the negative control. This introduces variability or non-linear dose response. DNA damage in cells exposed to fentanyl was significantly higher than in the negative control but lower than the positive control (H_2O_2) (Table 1). These parameters entered into Graphpad Prism 8 program and analyzed separately. Since the variance values between groups in the parameters were different, the Dunnet T3 test was chosen and comparisons were made with the control groups.

4. DISCUSSION

Since DNA can be damaged by various chemicals through different mechanisms, it is important reveal the genotoxicity of agents encountered by human via different genotoxicity test systems. In conclusion, under the conditions used in this study (short-term exposure), results indicate that evaluating the genotoxicity of fentanyl maintained anesthesia compared to the positive control is important,

considering dose and continuous exposure. It is crucial to conduct comet assay experiments with blood samples from patient profiles regularly exposed to long-term fentanyl exposure.

Fentanyl exposure induces DNA damage in healthy human lymphocytes, as shown via comet parameters. However, the genotoxic effect does not display a clear dose-dependent manner, suggesting that fentanyl may cause DNA damage through mechanisms that are not linearly dose-dependent manner. This effect at 20 µg/mL dose revealed nonlinear comet parameters. This dose response may suggest potential thresholds or cellular adaptation mechanisms.

Future research should focus on the specific pathways through fentanyl causes DNA damage. Additionally, it will be necessary to investigate in vivo whether the effects of fentanyl on DNA are reversible. In vivo evaluation of the long-term effects of repeated or chronic fentanyl exposure on DNA integrity would be very informative.

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CONFLICT OF INTEREST

Authors of the manuscript have no conflict of interest regarding the publication of the manuscript.

CRediT AUTHOR STATEMENT

Öge Artagan: Conceptualization, Research administration, Methodology, Investigation, Formal analysis, Data curation, Supervision, Writing the manuscript (**review and editing**), Validation. **Bahar Köklü**: Methodology, Investigation, Formal analysis, Visualization, Validation.& editing.

CONSENT OF THE PUBLICATION

Oge Artagan and Bahar Koklu agreed to publish the manuscript.

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