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INVESTIGATION OF THE GENOTOXIC EFFECTS OF THE ANALGESIC DRUGS PIROXICAM AND TENOXICAM IN HUMAN LYMPHOCYTES

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Abstract: Analgesics are one of the most widely used drug groups in the world. Since the genotoxic and carcinogenic effects of some analgesic drugs have been detected, studies investigating these effects have increased. In this study, the genotoxic effects of the analgesic drugs Tenoxicam and Piroxicam were investigated *in vitro* in human peripheral lymphocytes by chromosomal aberrations (CAs) and micronucleus (MN) assays. In addition, mitotic index (MI) and nuclear division index (NDI) values were also determined. As a result, no difference in chromosomal abnormalities was observed in both 24 and 48 hours of Piroxicam treatment. Micronucleus frequency was increased at the three highest concentrations (0.94, 1.88 and 3.75 µg/mL) compared to the control. Piroxicam significantly decreased MI compared to both control and solvent control at concentrations of 1.88 and 3.75 µg/mL at all exposure times. Tenoxicam showed a statistically significant increase at the highest concentration (10 µg/mL) compared to the control in the 24 h CA treatment. On the other hand, no effect was observed in 48-hour CA treatment. It was concluded that tenoxicam did not cause a toxic effect at any concentration except the highest concentration (10 µg/mL) in the MN assay results. Mitotic index (MI) significantly decreased at concentrations of 1.25, 2.5, 5 and 10 µg/mL. Nuclear division index (NDI) results did not change for both agents. In conclusion, both agents were found to be genotoxic only at high concentrations and the effect was weak. This study is pioneering as there have been no previous *in vitro* studies in human peripheral lymphocytes for both Piroxicam and Tenoxicam. These results need to be supported by different cell groups and *in vivo* assays.

Keywords: Piroxicam, Tenoxicam, Chromosomal aberrations, Micronucleus, Genotoxicity, Human lymphocytes

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1. Introduction

Pain is one of the most common conditions for which medication is preferred. Analgesic use is one of the leading pain treatment methods. They also known as painkillers, are the common name of the drug types preferred to provide pain relief. Analgesics are one of the most widely used drug groups in the world. They are used in the treatment of certain pains such as cancer pain, bone pain, neck pain and low back pain (Kılıc and Tuylu 2020). Analgesic drugs can be divided into six groups: opioids, pyrilazones, cannaboids, anilides, NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) and atypical drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to relieve pain, but they also have anti-inflammatory and antipyretic effects. Oxycams are in the NSAID group due to their chemical structure. They are analgesics used in the treatment of rheumatoid arthritis, osteoarthritis, acute gout and acute muscle pain. Their half-life is 20-60 hours. Since they are weakly acidic and easily absorbed in the stomach. They are usually transported by binding to albumin, a plasma protein (Yilmaz et al., 2017).

Piroxicam is an analgesic among the oxycams, a class of NSAIDs that are often preferred in the treatment of rheumatoid and ostoid arthritis because they have a relatively strong analgesic effect. Besides being analgesic, it also has antipyretic and anti-inflammatory effect. Piroxicam is a cheap and easily accessible drug. Although it has more side effects such as headache, dizziness and depression compared to other NSAIDs, its benefits outweigh these negative effects (Mirza et al., 2010; Shohin et al., 2014; Islami et al., 2020). Tenoxicam is also an oxycam and is a thionethionine derivative drug active substance in the NSAID group with analgesic, anti-inflammatory and platelet aggregation suppressing effect. Tenoxicam has been shown to act as a cofactor in reducing the activity of peroxidase in neutrophils, one of the blood cell groups, and this activity has been shown to be an anti-inflammatory property (Balkaya et al., 2021).

Genetic toxicity or genotoxicity is a general term covering damage to the nucleus, chromosome and DNA structure such as DNA insertions, DNA breaks, gene mutations,

chromosome abnormalities, clastogenicity and aneuploidy. Genotoxic substances that interact with enzymes that enable the copying of the genome DNA or cause mutation and damage to DNA or cause some changes are defined as genotoxins. Agents or mutagens that cause mutations in the DNA molecule show their effects on DNA either directly or indirectly by binding to proteins synthesised according to genomic information (Bagatir et al., 2022; Siivola et al., 2022).

Defects in key molecules and pathways involved in DNA damage lead to tissue damage, ageing, cancer, infertility and some genetic and multifactorial diseases. The relationship between genotoxicity and carcinogenicity was examined in many studies and it was shown that many compounds that are carcinogenic to humans are genotoxic. The demonstration of a strong correlation between the mutagenic effects of chemical substances and their carcinogenic potential has led to the use of genotoxicity tests as screening tests by industrial organisations to investigate the carcinogenic risks of chemical substances (Alonso-Jauregui et al., 2023; Sherif et al., 2023). For this purpose, Chromosomal abnormality and Micronucleus tests are frequently preferred tests for genotoxicity evaluations. Chromosomal abnormalities are usually caused by a failure to separate chromosomes correctly during division and/or a failure to repair errors in the chromosomes. Epidemiological studies show that an increase in the frequency of errors occurring in chromosomes can lead cells to cancer (Hagmar et al., 2004; Mamur et al., 2018). Micronuclei are small nuclei formed during mitosis in cells, which are not attached to the nucleus, originating from a whole chromosome or an acentric chromosome fragment. It is preferred for the detection of carcinogens in cultured human lymphocytes. Further studies allow this test to be used in the determination of agents causing aneuploidy (Ustuner, 2011; Yuzbasioglu and Avuloglu Yilmaz, 2022).

In this study, the possible genotoxic potentials of Piroxicam and Tenoxicam were examined by two genotoxicity tests, chromosomal abnormality and micronucleus tests, which have proven to be reliable and sensitive. Both test systems (*in vitro* chromosomal aberration assay test number: 473, *in vitro* micronucleus assay test number: 487) were accepted by the OECD for their validity and safety in testing chemicals and performed for many years (OECD, 2016a; OECD, 2016b).The aim of this study was to investigate the genotoxic effects of the commonly used analgesic drugs active ingredients Piroxicam and Tenoxicam. There is no study investigating the genotoxic effects of these two active ingredients in human peripheral lymphocytes. For this purpose, chromosomal aberration and micronucleus assays were performed in *in vitro* human peripheral lymphocytes.

2. Materials and Methods

Cultured human peripheral lymphocyte cells were used to evaluate the genotoxic effects of Piroxicam and Tenoxicam. For this purpose, peripheral blood was collected from two female donors who had no health problems, did not smoke or drink alcohol, were between 18-25 years of age and had not been exposed to any genotoxic agent for at least three months. Drug active ingredients were purchased from Sigma-Aldirich.

2.1. Determination of concentrations

In this study, in determining the concentrations used in both Chromosomal Aberrations (CA) and Micronucleus (MN) tests, the LD50 concentrations of the substance determined in the studies conducted with experimental animals in the literature were taken as reference. Based on these concentrations, preliminary experiments were conducted to determine the concentrations to be used in the study. Piroxicam concentrations of 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15, 30, 60, 120 µg/mL were tested and 0.23, 0.47, 0.94, 1.88, 3.75 µg/mL concentrations were selected. Tenoxicam concentrations of 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40 µg/mL were tested and concentrations of 0.63, 1.25, 2.5, 5, 10 µg/mL were selected.

2.2. Chromosomal aberration assay

The chromosomal aberration (CA) assay was performed according to various revisions (Yuzbasioglu et al., 2006) of the method of Evans et al. (1984). Heparinised blood obtained from donors was added to tubes containing of medium. The cells were treated with the above-mentioned concentrations of Piroxicam and Tenoxicam after 24 and 48 hours after the start of culture. On the other hand, distilled water was used as negative control and Mitomycin-C (MMC) at a concentration of 0.20 µg/mL was used as positive control. DMSO (4.81 µg/mL) and methanol (3.70 µg/mL) were used as solvent control for Piroxicam and Tenoxicam respectively. At 70 h after the start of culture, 0.06 µg/mL colchicine solution was added to each tube. The purpose of this procedure is to pre-treat the cells. At the end of the 72nd hour, culture tubes were centrifuged at 1200rpm for 10 minutes, KCl (0.075M) was added to the tubes and incubated at 37°C for 30 minutes. Then, centrifugation was performed again. Cells were fixed with 3:1 methanol: acetic acid mixture at +4°C for 45 minutes. Afterwards, this cold fixation process was repeated for 2 times. Cells remaining at the bottom of each tube were homogenised by pipetting. These suspensions were spread by dropping onto clean, cooled slides. The dried slides were stained with 5% Giemsa and fixed with entallan.

For the determination of chromosome aberrations, a total of 400 metaphases (200 metaphases from each donor) with well observable chromosomes were analysed for each concentration. The percentage of abnormal cells among the analysed metaphases and the chromosome abnormality per cell were determined. Quantification of mitotic index (MI), 2000 cells from the slides prepared for all concentrations (4000 cells in total) were analysed. Mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells in percentage.

2.3. Micronucleus assay

Micronucleus (MN) test was performed according to various revisions of the method of Fenech et al. (2000). For this purpose, firstly, heparinised blood was collected from each donor to prevent clotting and 0.2 mL was added to each tube containing medium. These tubes were incubated at 37°C for 72 hours. At the 48th hour of culture, the cells in culture were treated with the previously mentioned concentrations of Piroxicam and Tenoxicam. In addition, negative, positive and solvent control groups were maintained at the concentrations described in the chromosomal aberration assay. At 44 h, Cytochalasin-B (5.2 µg/ml) was added to the culture to inhibit cytokinesis. After 72 hours, each tube was centrifuged at 1000 rpm and the supernatants were removed. KCl solution was added to the remaining part and centrifugation was repeated. Fixative (3:1 methanol:acetic acid) was added to the culture tubes and they were kept at +4 for 15 min. This process was repeated twice and formaldehyde was added to the fixative solution in the last treatment. Then centrifugation was performed for the last time. The supernatant in each tube was removed, the cells were homogenised and spread on the cold slides which were previously cleaned. Slides was allowed to dry at room temperature and stained with Giemsa (5 % pH:6.8).

For the determination of micronuclei, 2000 binucleates were counted from each slide (total 4000) for each concentration. The formula [1x(1MN)+2x(2MN)+3x(3MN+4MN)]/N was used for MN/Cell. Here, N refers to the total number of cells analysed.

Nuclear division index (NDI) was determined by counting 1000 cells from each donor at each concentration and 2000 cells in total. NDI was calculated according to the formula [1x(1N)+ 2x(2N)+3x(3N+4N)]/n. n refers to the total number of cells and (1N) mononuclear, binuclear (2N), trinuclear (3N) or tetranuclear (4N) cells.

3. Results

3.1. The results of Piroxicam treatment

Piroxicam treatment of CA results is given in Table 1 and it was observed that it did not significantly increase the percentage of abnormal cells at any concentration compared to the control at both 24 and 48 hours. Similarly, the number of abnormalities per cell was not statistically increased at any concentration compared to both control and solvent control (Table 1). The structural abnormalities detected on cultured human lymphocytes for 24 and 48 hours' treatments were chromatid break (29.82%), chromosome break (25.44%), sister chromatid union (9.65%), dicentric chromosome (15.79%), fragment (4.39%) and chromatid exchange (14.91%). No numerical abnormality was observed as a result of piroxicam treatment.

Piroxicam significantly decreased the mitotic index at the two highest concentrations (1.88 and 3.75 µg/mL) compared to the control and solvent control at both treatment times (Table 2). This decrease was not concentration dependent (r= - 0.14 for 24 h, r= - 0.24 for 48 h).

Piroxicam increased the MN frequency at the three highest concentrations (0.94, 1.88 and 3.75 µg/mL) compared to the negative control with concentration-depend (r=0.96). No statistical difference was detected in MN frequency compared to the solvent control. Additionally, Piroxicam did not cause any change in NDI (Table 3).

3.2. The results of Tenoxicam treatment

The CA test results of Tenoxicam are given in Table 4. Tenoxicam treatment significantly increased the percentage of abnormal cells at 24 hours compared to the negative control only at the highest concentration (10 µg/mL). Similarly, the number of abnormalities per cell was significantly increased only at the highest concentration (10 µg/mL) compared to both negative and solvent controls. These increases were concentration dependent (percentage of abnormal cells and abnormalities per cell for 24 hours r=0.80). On the other hand, 48 hours of Tenoxicam treatment did not significantly affect the frequency of chromosomal abnormalities and percentage of abnormal cells per cell at any concentration. Tenoxicam was induced six different structural abnormalities in human lymphocytes at 24- and 48-hours treatments (chromatid break (34%), chromosome break (26%), sister chromatid union (24%), dicentric chromosome (8%), fragment (6%) and chromatid exchange (2%)).

Tenoxicam decreased the mitotic index at all concentrations except the lowest concentration (0.63 µg/mL), however, this decrease was not concentration dependent (r=-0.31) in 24-h treatment compared to the negative control. In addition, mitotic index decreased significantly at the three highest concentrations compared to the solvent control. In 48 h treatment, similarly, mitotic index decreased significantly compared to the negative control at all concentrations except the lowest concentration (0.63 µg/mL). However, there was a significant decrease only at the highest concentration compared to the solvent control. The correlation between the decrease in MI values and concentrations was weak (r=-0.42) (Table 5).

Tenoxicam statistically increased the micronucleus frequency at the three highest concentrations (2.5, 5 and 10 µg/mL) compared to the negative control, but only at the highest concentration compared to the solvent control. In addition, these increases were concentration dependently (r= 0.95). It was determined that Tenoxicam treatment did not affect NDI values (Table 6).

Table 1. Chromosomal abnormalities in human peripheral lymphocytes induced by piroxicam

Test substance	Treatment		Abnormalities						Abnormal cell ± SE (%)	CA/cell ± SE
	Period (hour)	Concentration (µg/mL)	ctb	csb	scu	dic	ex	f		
Control	24	0.00	5	-	-	-	2	-	1.5±0.60	0.0175±0.0489
MMC	24	0.20	6	2	1	6	65	1	17.5±1.89	0.2025±0.2213
DMSO	24	4.81 (µl/mL)	1	1	1	2	3	-	1.75±0.64	0.0200±0.0700
Piroxicam	24	0.23	1	2	-	2	1	1	1.25±0.54	0.0175±0.0489
		0.47	1	2	-	3	3	-	2.25±0.73	0.0220±0.0700
		0.94	2	3	1	3	1	1	2.25±0.73	0.0275±0.0700
		1.88	3	4	1	3	1	1	2.75±0.81	0.0325±0.0860
		3.75	4	4	3	2	-	-	2.25±0.73	0.0325±0.0860
Control	48	0.00	3	1	1	-	2	1	2.00±0.70	0.0200±0.0700
MMC	48	0.20	3	2	1	8	69	2	18.25±1.92	0.2125±0.2236
DMSO	48	4,81 (µl/mL)	2	4	1	-	5	1	2.75±0.81	0.0322±0.0860
Piroxicam	48	0.23	2	2	-	1	1	-	1.50±0.60	0.0150±0.0489
		0.47	3	4	1	1	2	1	1.75±0.64	0.0300±0.0860
		0.94	3	3	1	-	2	-	2.25±0.73	0.0222±0.0700
		1.88	3	3	2	2	1	-	2.50±0.77	0.0275±0.0700
		3.75	4	2	2	1	5	1	3.00±0.84	0.0375±0.0860
Frequency of abnormalities (%)			29.82	25.44	9.65	15.79	14.91	4.39		

ctb= chromatid break, csb= chromosome break, scu= sister chromatid union, dic= dicentric chromosome, ex= chromatid exchange, f= fragment.

Table 2. Mitotic index values observed in lymphocytes with piroxicam treatment

Test substance	Treatment		MI
	Period (hour)	Concentration (µg/mL)	MI±SE %
Control	24	0.00	6.78 ± 0.40
DMSO	24	4.81 (µl/mL)	6.53 ± 0.39
MMC	24	0.20	3.25 ± 0.28
Piroxicam	24	0.23	6.88 ± 0.40
		0.47	6.48 ± 0.39
		0.94	5.75 ± 0.37
		1.88	5.25 ± 0.35**a_1
		3.75	2.53 ± 0.25***a_3
Control	48	0.00	6.68 ± 0.39
DMSO	48	4.81 (µl/mL)	6.28 ± 0.38
MMC	48	0.20	3.00 ± 0.27
Piroxicam	48	0.23	6.55 ± 0.39
		0.47	6.23 ± 0.38
		0.94	5.68 ± 0.37
		1.88	5.20 ± 0.35** a_1
		3.75	3.18 ± 0.28***a_3

* Significantly different from the negative control P<0.05 (z test), ** Significantly different from the negative control P<0.01 (z test), *** Significantly different from the negative control P<0.001 (z test), a_1Significantly different from the solvent control P<0.05 (z test), a_2 Significantly different from the solvent control P<0.01 (z test), a_3 Significantly different from the solvent control P<0.001 (z test).

Table 3. Effect of piroxicam on micronucleus frequencies and nuclear division index in human lymphocytes

Test substance	Treatment		Binucleated cells (BN) scored	Distribution of BN cells according to the number of MN			MN ± SE (%)	Nuclear division index (NDI) ± SE
	Period (hour)	Concentration (µg/mL)		(1)	(2)	(3)		
Control	48	0.00	4000	19	-	-	0.48±0.11	0.73±0.19
MMC	48	0.20	4000	138	-	-	3.45±0.30	0.67±0.18
DMSO	48	4.81 (µl/mL)	4000	30	-	-	0.75±0.14	0.69±0.18
Piroxicam	48	0.23	4000	18	-	-	0.43±0.10	0.75±0.19
		0.47	4000	25	-	-	0.63±0.12	0.76±0.19
		0.94	4000	39	-	-	0.98±0.16**	0.74±0.19
		1.88	4000	43	-	-	1.08±0.16**	0.74±0.19
		3.75	4000	45	-	-	1.13±0.17***	0.77±0.19

** Significantly different from the negative control P<0.01 (z test), *** Significantly different from the negative control P<0.001 (z test).

Table 4. Chromosomal abnormalities in human peripheral lymphocytes induced by tenoxicam

Test substance	Treatment		Abnormal cell ± SE (%) Abnormalities							CA/cell ± SE
	Period (hour)	Concentration (µg/mL)	ctb	csb	scu	dic	ex	f		
Control	24	0.00	3	-	-	-	-	-	0.50 ±0,353	0.0075±0.0043
MMC	24	0.20	31	18	10	11	-	3	17.25±1.890	0.1825±0.0193
Methanol	24	3.70 (µl/mL)	3	-	-	3	-	-	1,00 ±0.498	0.0150±0.0061
Tenoxicam	24	0.63	-	1	-	-	-	-	0.25 ±0.250	0.0025±0.0025
		1.25	-	2	-	-	-	-	0.50 ±0.353	0.0050±0.0035
		2.50	-	-	1	1	-	-	0.25 ±0.250	0.0050±0.0035
		5.00	1	2	1	-	-	-	0.75 ±0.431	0.0100±0.0048
		10.00	6	2	1	1	-	3	2.50 ±0.781*	0.0325±0.0089*c
Control	48	0.00	3	1	1	-	-	-	1,00±0.498	0.0125±0.0055
MMC	48	0.20	37	10	21	5	-	-	16.25±1.84	0.1825 ±0.0193
Methanol	48	3.70 (µl/mL)	-	-	-	2	5	-	1.25±0.550	0.0175±0.0065
Tenoxicam	48	0.63	-	-	1	-	-	-	0.25±0.062	0.0025±0.0024
		1.25	1	1	2	-	-	-	0.75±0.431	0.0100±0.0350
		2.50	1	1	1	1	-	-	0.75±0.431	0.0100±0.0350
		5.00	2	2	2	-	-	-	1.50±0.607	0.0150±0.0060
		10.00	3	2	3	1	1	-	1.75±0.429	0.0250±0.0078
FA (%)			34.00	26.00	24.00	8.00	2.00	6.00		

FA= frequency of abnormalities, ctb= chromatid break, csb= chromosome break, scu= sister chromatid union, dic= dicentric chromosome, ex= chromatid exchange, f= fragment.

* Significantly different from the negative control P<0.05 (z test), cSignificantly different from the solvent control P<0.001 (z test)

Table 5. Mitotic index values observed in lymphocytes with tenoxicam treatment

Test substance	Treatment		MI
	Period (hour)	Concentration (µg/ml)	MI±SE %
Control	24	0.00	7.00 ± 0.40
DMSO	24	3.70 (µl/mL)	6.68 ± 0.39
MMC	24	0.20	3.40 ± 0.29
Tenoxicam	24	0.63	6.50 ± 0.39
		1.25	5.80 ± 0.37*
		2.50	5.58 ± 0.36**a_1
		5.00	5.23 ± 0.35**a_2
		10.00	3.63 ± 0.30***a_3
Control	48	0.00	7.25 ± 0.41
DMSO	48	3.70 (µl/mL)	6.38 ± 0.39
MMC	48	0.20	3.90.± 0.31
Tenoxicam	48	0.63	6.25 ± 0.38
		1.25	5.88 ± 0.37*
		2.50	5.60 ± 0.36**
		5.00	5.48 ± 0.36**
		10.00	4.20 ± 0.32*** a_3

* Significantly different from the negative control P<0.05 (z test), ** Significantly different from the negative control P<0.01 (z test), *** Significantly different from the negative control P<0.001 (z test), a_1Significantly different from the solvent control P<0.05 (z test), a_2 Significantly different from the solvent control P<0.01 (z test), a_3 Significantly different from the solvent control P<0.001 (z test).

Table 6. Effect of tenoxicam on micronucleus frequencies and nuclear division index in human lymphocytes

Test substance	Treatment		Binucleated cells (BN) scored	Distribution of BN cells according to the number of MN			MN ± SE (%)	Nuclear division index (NDI) ± SE
	Period (hour)	Concentration (µg/mL)		(1)	(2)	(3)		
Control	48	0.00	4000	21	-	-	0.53 ± 0.11	0.71 ± 0.18
MMC	48	0.20	4000	130	-	-	3.25 ± 0.28	0.65 ± 0.18
Methanol	48	3,70 (µl/mL)	4000	27	-	-	0.68 ± 0.13	0.70 ± 0.18
Tenoxicam	48	0.63	4000	19	-	-	0.48 ± 0.11	0.67 ± 0.18
		1.25	4000	22	-	-	0.55 ± 0.12	0.69 ± 0.18
		2.50	4000	40	-	-	1.00 ± 0.16*	0.66 ± 0.18
		5.00	4000	36	-	-	0.90 ± 0.15*	0.66 ± 0.18
		10.00	4000	47	-	-	1.18 ±0.17**a_1	0.73 ± 0.19

* Significantly different from the negative control P<0.05 (z test), ** Significantly different from the negative control P<0.01 (z test), a_1Significantly different from the solvent control P<0.05 (z test).

4. Discussion

In this study, CA and MN tests were performed in human lymphocytes to determine the *in vitro* genotoxic effects of Piroxicam and Tenoxicam, the active ingredients of NSAIDs analgesic drugs. NSAIDs show their effects through COX (cyclooxygenase) enzymes. As a result of the translation from arachidonic acid to prostoglandin, they act by non-selectively inhibiting COX enzymes. NSAIDs inhibit prostoglandin synthesis after tissue damage. These drugs are needed to prevent both pain and inflammation in damaged tissues. NSAID group analgesics have significant side effects. The most frequently observed side effect of this group of drugs is on the gastrointestinal system due to inhibition of COX enzymes. NSAIDs provide reversible or irreversible inhibition of COX by competing with arachidonic acid for the active site of the enzyme. (Himly et al., 2003; Becker and Phero, 2005; Ali et al., 2023)

CA and MN test results showed that piroxicam did not induce the formation of chromosomal abnormalities but induced micronucleus formation at the three highest concentrations (0.94, 1.88, 3.75 µg/mL). The other analgesic Tenoxicam, whose genotoxic potential was examined in the study, is induced chromosomal abnormality formation only at the highest concentration (10 µg/mL) and induced micronucleus formation at concentrations of 2.5, 5 and 10 µg/mL. In addition, Piroxicam was found to decrease mitotic index at the two highest concentrations and Tenoxicam caused a significant decrease in mitotic index values. However,

nuclear division index (NDI) did not change for treatment of both analgesics.

The concept of genotoxicity refers to damage to the structure or quantity of chromosomes or DNA caused by any physical, biological or chemical agent. The chromosomal aberration (CA) assay detects the structure and number of any changes that occur in the chromosome or in the sister chromatids that make up the chromosome. Micronuclei are caused by defects in kinetochore and spindle threads during the karyokinesis stage of cell division, deficiencies in genes that control cell division, and finally damage to chromosomes (Bonassi et al., 2011). Cytogenetic modifications, which are changes in chromosome structure and number, have been described as the types of genetic changes that contribute to the development and progression of cancer (Norppa et al. 2006, Ewing et al., 2023). Furthermore, Micronuclei (MNi) and other nuclear abnormalities such as nucleoplasmic bridges (NPBs) and (NBUDs) are known to be biomarkers of genotoxicity and indicators of chromosomal instability that often occur in cancer (El-Zein et al., 2011, Raj and Rajitha, 2023).

Exposure to biological, physical and chemical agents causes differences in either the structure or the number of chromosomes. Structural abnormalities are chromatid breaks, chromosome breaks, fragments, dysentric chromosomes, sister chromatid unions. Polyploidy is an example of numerical abnormalities. If the effect of the chemical occurs in the first phase of interphase (G1), chromosome-type abnormalities; if it occurs in the second phase (G2), chromatid-type abnormalities; if it occurs in the synthesis phase (S), both types of abnormalities may be observed. Chromosomal abnormalities are usually caused by the misjoining or non-joining of chromosome ends originating from DNA single and double strand breaks. As expected, as the number of DNA strand breaks increases, the number of misjoins or failures to join may increase. The formation of two breaks on two different chromosomes can result in a dyscentric chromosome with two centromeres, or an acentric fragment with reciprocal translocation where both chromosomes have one centromere. On the other hand, fragments are also known to be formed as a result of breaks in chromosomes or chromatids as well as terminal deletions (Natarajan and Boei, 2003; Tımoroglu et al., 2014; Ila and Husunet, 2022).

Damages occurring in the genome are effective in the occurrence of micronuclei. Clastogenic, mutagenic or carcinogenic factors are important in the formation of these damages. In micronuclei caused by any reason, the damage that has occurred anywhere along the genome cannot be repaired or even if it is repaired, it may not be fully repaired. In addition, mutations in kinetochores, which are special proteins, or in centromeres that join chromatids or in spindle threads originating from the centrosome organelle can also cause micronucleus formation. However, errors can also occur in the genes responsible for the repair of these errors in DNA. In such a case, an increase in the frequency of micronucleus formation is an expected result since repair of errors is not possible (Fenech, 1993; Choy, 2001; Ellwanger et al., 2023). Although piroxicam did not induce chromosomal abnormalities, it may have increased the frequency of MNs due to the failure to repair defects in mitotic apparatus, spindle threads or centrosome responsible for spindle thread formation and centrioles originating from centrosome. In addition, defects in repair mechanisms may not have been repaired and the cell may have lost its viability spontaneously (Fenech, 2000; Ellwanger et al., 2023).

Mitotic Index (MI) is used to evaluate the cytotoxicity of different chemical agents. It is a preferred parameter to obtain information about the mitosis process. Most chemical agents that cause cytotoxicity cause a decrease in mitotic index values. The reason for this decrease in mitotic index is the inhibition of the G2 phase, which initiates mitosis of cells, and the suppression of enzymes involved in DNA replication and forming spindle threads. The decrease in mitotic index is also caused by the loss of the cell's ability to divide and the presence of factors that cause cell death (Van't Hof, 1968; Jain and Sorbhoy, 1988; Takebayashia et al., 2023). In this study, the decrease in MI values of both drug active ingredients, except at low concentrations, indicates that cell cycle progression is prevented and/or capacity is reduced (Riss and Moravec, 2004).

In order to examine the genotoxic effects of drug active ingredients, the guidelines established by the Organisation for Economic Cooperation and Development (OECD) for the testing of chemicals are considered. Genotoxicity tests that should be performed if the active substance is intended to be placed on the market are gene mutation test in bacteria, cytogenetic examination of *in vitro* chromosomal damage in mammalian cells or gene mutation test in *in vitro* mammalian cells and lastly *in vivo* chromosomal damage test using rodent haematopoietic cells (OECD, 2008). However, the lack of genotoxicity studies on Piroxicam and Tenoxicam drug active ingredients is noteworthy. This is the first study to investigate the genotoxicity of both drug active ingredients in human lymphocytes *in vitro.*

Since some analgesic drug active ingredients were found to have genotoxic and carcinogenic effects, the number of studies examining the effects of these substances were increased. In order to investigate the genotoxicity of Piroxicam, *in vitro* MN assay (50, 1000 and 1500 µg/mL) was performed in mouse, rat, monkey, dog and human liver cells. The results showed that Piroxicam did not increase the MN frequency and therefore did not show any genotoxic effect (Kishino, et al., 2019). The genotoxic effect of piroxicam was investigated in 20-25 g healthy *Mus musculus* treated at a dose of 0.3 mg/kg for 24 hours by CA assay. At the same time, cytotoxicity was also evaluated using mitotic index. As a result, no significant increase in chromosomal abnormalities and no change in

mitotic index frequency were observed (Dkhil et al., 2011).

RAW 264.7 macrophages were treated with Piroxicam and liposome-coated Piroxicam concentrations of 0.1, 0.2 and 0.4 mg/ml for 24 hours and cytotoxicity was evaluated using MTT assay. It was observed that Piroxicam treatments did not significantly decrease cell viability, while liposome-coated Piroxicam significantly decreased cell viability at the highest concentration (Chiong et al., 2013). In patients exposed to tenoxicam, piroxicam and lornoxicam at a dose of 20 mg/day for 2 weeks, the in vivo frequency of sister chromatid exchanges (SCEs) was increased, except in piroxicam-treated patients. It was indicated that treatment with oxycams may indicate a genotoxic risk due to elevated SCE levels (Kullich and Klein 1986; Brambilla and Martelli, 2009). Ames test was performed with *Bacillus subtilis*, *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and *E. coli* WP2 uvrA strain with 1000 g/plate concentration of tenoxicam and it was reported that it did not induce mutation (Brambilla and Martelli, 2009).

Genotoxicity studies with Piroxicam and Tenoxicam are very limited. However, studies investigating the genotoxic potential of different analgesic drug active ingredients were encountered. The genotoxicity of ibuprofen (oral doses of 10, 20, 40 and 60 mg/kg) was investigated in mice by in *vivo* CA assay. It was observed that mitotic index values decreased at doses of 40 and 60 mg/kg and chromosomal abnormality frequencies increased dose-dependently in bone marrow cells of mice (Rina et al., 2012). In another study, the genotoxicity of dexketoprofen trometamol in human lymphocytes was investigated by MN and comet assays. For this, cells were exposed to concentrations in the range of 100-1000 mg/ml for 24 and 48 hours. As a result, it was observed that MN frequency and comet tail length and intensity increased especially at high concentrations (Kılıc and Tuylu, 2020).

The effects of repeated doses of meloxicam, an NSAID from the oxycam group, on genotoxicity, oxidative stress and histopathological changes in mice were evaluated. For this purpose, 0.1, 0.5 and 1 mg/kg meloxicam were given to animals for 28 days. The highest concentration was not change MN frequency but induced DNA damage in comet test. Meloxicam did not affect catalase activity but increased plasma ferric reduction capacity (FRAP) (1 mg/kg). It was evaluated that meloxicam could potentially cause cardiac pathological changes and genotoxic effects (da Silva et al., 2022) These effects can be explained by the induction of ROS formation by anti-inflammatory drugs and analgesics. Although studies indicate that NSAIDs reduce ROS formation by inhibition of COX enzyme, it is also a fact that ROS production caused by them endangers DNA integrity. Studies in the literature show that especially prolonged exposure to NSAIDs may have genotoxic effects (Bhattacharya et al.2000; Fernandes et al.2003; Pandey and Rizvi 2010; da Silva et al., 2022). The genotoxic effects of Piroxicam and Tenoxicam observed in this study may be due to aromatic amines. Despite their known and proven mutagenicity and carcinogenicity, aromatic amines are frequently found in drug molecules, including oxycams, due to their usefulness in synthesis (Harding et al., 2015). Additionally, oxycams are characterized by the presence of the 4-hydroxybenzothiazine heterocycle. Piroxicam, known to have gastrointestinal toxicity in vivo, did not show significant PGHS-1 selectivity in vitro. The cytochrome P450 (CYP) oxidase system is responsible for most NSAID metabolism and CYP2C9 is the most important oxidase primarily responsible for the metabolism of a wide range of NSAIDs, including piroxicam and tenoxicam (Bindu et al., 2020).

In this study, the genotoxic effects of Piroxicam and Tenoxicam, the active ingredients of analgesic drugs, were investigated by performed chromosomal aberrations and micronucleus assays. It was determined that the risk of genotoxic effects increased as the concentration increased in different test systems of both active substances and similar results were valid for cytotoxicity. However, when the data obtained are evaluated collectively, it is possible to state that Piroxicam and Tenoxicam exhibit a weak genotoxic effect. It is thought that *in vivo* studies, especially for high concentrations, will contribute to the understanding of their genotoxic potential and mechanisms of action.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	H.G.	E.A.Y.
C	50	50
D	50	50
S	20	80
DCP	80	20
DAI	60	40
L	70	30
W	20	80
CR	20	80
SR	10	90
PM	50	50
FA	30	70

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

This study was conducted with the permission of Amasya University Clinical Ethics Committee (approval date: 3 June 2021 and protocol code: 86). The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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