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Original Article

Genotoxic of co-codamol for human lymphocyte culture *in vitro*

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Abstract

Co-codamol, a combination analgesic containing paracetamol and codeine phosphate, is widely used for pain relief, but its potential genotoxic effects on human lymphocytes remain largely unknown. This in vitro study investigates the genotoxic potential of co-codamol on cultured human lymphocytes by assessing cell viability, mitotic index (MI), chromosomal aberration frequency, and micronucleus (MN) formation. Lymphocytes were exposed to varying concentrations of co-codamol (0.02-0.12 mg/mL), and cytotoxicity was determined using the MTT assay. Results showed that co-codamol significantly reduced cell viability in a dose-dependent manner, with complete cell death at 0.12 mg/mL. The mitotic index was significantly decreased at higher concentrations, and a statistically significant increase in chromosomal aberrations and micronucleus formation was observed in treated lymphocytes compared to the control group ($p < 0.05$). These findings provide important evidence that co-codamol exhibits genotoxic potential in vitro, suggesting a potential risk of DNA damage associated with its use. Further in vivo investigations are warranted to assess the clinical relevance of these genotoxic effects and to elucidate the underlying mechanisms of toxicity.

Keywords: Co-codamol, Genotoxicity, Human lymphocyte culture, MTT.

1. Introduction

Co-codamol contains paracetamol and codeine phosphate, which is a compound of two compounds [1]. The first is paracetamol, which is a widely used pain reliever for mild pain[2]. There are studies and research, although few, that have shown its effect on living cells [3-5]. The second is codeine phosphate: a pain reliever from the opioid family, sometimes called a narcotic, which does not treat the cause but rather reduces the symptoms, such as coughing in the case of bronchitis, for example [6] Codeine works by “changing the brain and nervous system's response to pain, by decreasing the activity of the part of the brain that causes pain”. Both are pain relievers combined to form co-codamol, which has become a pain reliever for severe pain, such as surgery, migraines, etc. However, using it in high doses for several weeks or more can damage the liver and kidneys, and sometimes stomach ulcers, especially when combined with a large consumption of ethanol [6-8]. Co-codamol raises many serious concerns when used incorrectly or for a long period in high doses. Other risk factors for co-codamol use, such as genetics or genotoxicity, have been suggested. To assess the potential for co-codamol to cause mild genetic damage [8]. Co-codamol is commonly used in medical practice to treat mild to moderate pain, especially when regular analgesic therapy has failed. It is “thought to work by blocking signals from nerves to the brain. Potentially severe side effects of co-codamol include hypersensitivity”, liver damage, low white blood cell counts, and addiction. Several serious concerns have also been highlighted regarding co-codamol, including incorrect use or high long-term doses. Other contributing factors have been suggested for co-codamol use, such as genetics or genotoxicity [9]. Lymphocytes are established reference cells for monitoring chromosomal instability and predicting cancer. Consequently, most knowledge about mutation has been generated from this model, which is typically performed using lymphocyte cultures in a metabolic-free in vitro system [10]. The MTT (dimethyl thiazolyl diphenyl tetrazolium salt) test is the most popular test for checking cell viability [11]. The objective of this drug toxicity audit, or other supplements on the cell. A micronucleus is a broken part of a chromosome or rarely a whole chromosome that remains outside the nucleus after cell division. A micronucleus may form after direct DNA damage (DNA breakage mechanism) or after a secondary interaction with the DNA replication apparatus (indirect regenerative mechanism) [12-14]. The current aims are to identify the defect in the genetic material of cultured lymphocytes by studying the division rate, chromosomal abnormalities, and micronuclei and to evaluate the possibility of co-codamol in causing such genetic damage.

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2. Materials and methods

2.1. Chemicals and reagents

Co-codamol tablets containing 500 mg paracetamol and 8 mg codeine phosphate (500/8) were used in this study. The method was conducted according to Wang et al. (2006) [15]. The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), Lymphoprime medium (without phenol red), colchicine, cytochalasin B, hypotonic potassium chloride solution (0.075 M, 0.45%), glacial acetic acid, methanol, and Giemsa stain were obtained from standard suppliers. All chemicals were of analytical grade and prepared according to the manufacturers' instructions.

2.2. Preparation of Co-codamol solutions

Stock solutions of co-codamol were prepared by dissolving the tablets in an appropriate solvent to achieve final concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg/mL in Lymphoprime medium. These concentrations were selected based on the standard adult dose and pharmacological relevance. Each concentration was added to the lymphocyte cultures in duplicate tubes.

2.3. Lymphocyte culture

Peripheral blood samples were collected and cultured in Lymphoprime medium under sterile conditions. The cultures were incubated at 37 °C for 72 hours. The cell density was adjusted to 0.1–0.5 × 10^5 cells/mL, and each well of a 96-well plate received 150 µL of cell suspension. Co-codamol was added at the specified concentrations, and cultures were maintained under standard conditions.

2.4. MTT cytotoxicity assay

Cell viability was assessed using the MTT cytotoxicity assay according to Wang et al. (2006) [15]. MTT was prepared at 5 mg/mL in Lymphoprime medium (1:10), filtered through a 0.2 µm membrane, and stored at 4–6 °C. After 44 hours of incubation with co-codamol, 20 µL of MTT solution was added to each well 4 hours before the end of incubation. The cultures were transferred to Eppendorf tubes, centrifuged at 500 rpm for 15 minutes, and the supernatant was removed. DMSO (100 µL) was added to dissolve the formazan crystals, and absorbance was measured at 560 nm using a spectrophotometer. Cytotoxicity was calculated using the formula:
Inhibition (%) = [1 - (O.D of Test / O.D of Control)] × 100.

2.5. Assessment of mitotic index and chromosomal aberrations

After 72 hours of culture, colchicine (0.1 µg/mL) was added to one set of cultures two hours before harvesting to arrest cells in metaphase. Cells were centrifuged at 1000 rpm for 10 minutes at 37 °C, resuspended in hypotonic KCl solution for 15 minutes, and fixed three times with glacial acetic acid and methanol (1:3, v/v). Cell suspensions were dropped onto clean slides, air-dried, and stained with 10% Giemsa for 15 minutes. Slides were examined under a microscope at 1000× magnification. A total of 1000 lymphocytes per culture were analyzed. The mitotic index (MI) was calculated as:
%MI = (No. of Dividing Cells / No. of Dividing and Non-dividing Cells) × 100
Fifty mitotic figures per sample were evaluated for chromosomal aberrations, including gaps and other structural changes [16-20].

2.6. Micronucleus assay

To assess micronuclei formation, cytochalasin B (0.5 µg/mL) was added to a second set of replicate cultures at 48 hours. After incubation, the same procedures for centrifugation, hypotonic treatment, fixation, and slide preparation were followed. The percentage of micronucleated cells was determined by scoring 1000 lymphocytes per culture under a microscope [16-20].

2.7. Statistical analysis

All data were analyzed using IBM SPSS statistical software for Windows. Results were expressed as mean ± standard deviation (SD). Comparisons between groups were performed using independent sample t-tests, and differences were considered statistically significant at $p < 0.05$ or $p < 0.01$.

3. Results

Calculation of viability of living cells with increasing concentration of co-codamol and interpretation of the results in Table 1. The relationship between increasing concentration of the drug and viability of living cells is shown in Figure 1. Percentage of co-codamol in cell density, Figure 2. Cells exposed to a higher concentration of cytotoxicity. The cytotoxicity of the preparation is high with increasing concentrations of the drug. MTT test was performed to examine the cytotoxicity of this drug. The results of the test showed cytotoxicity with increasing concentration. All cells died at high a concentration of co-codamol (0.12 mg/ml). The percentage of living cells at

Table 1. Viable cell counts and viability percentage after treatment with Co-codamol.

Control &conce. Of Co-codamol	Co-codamol (mg/ml)	The volume of cells (ml)	number from the cells /20µl	The number from the viable cells/20µ	proportion of survival
control	0.0	5.0	260	240	92
1	0.02	5.0	225	200	88.9
2	0.04	5.0	200	160	80
3	0.06	5.0	150	110	73.3
4	0.08	5.0	100	70	70
5	0.10	5.0	50	20	40
6	0.12	5.0	20	0	0

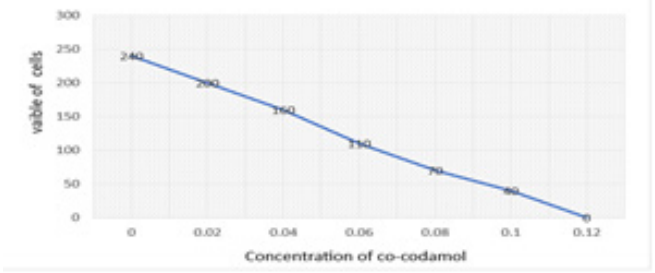

Fig. 1. Dose-Dependent Reduction in Cell Viability of Human Lymphocytes Following Co-codamol Exposure. Relationship between increasing concentrations of co-codamol (0.02–0.12 mg/mL) and the percentage of viable human lymphocytes, as determined by cell counting after 44 hours of incubation.

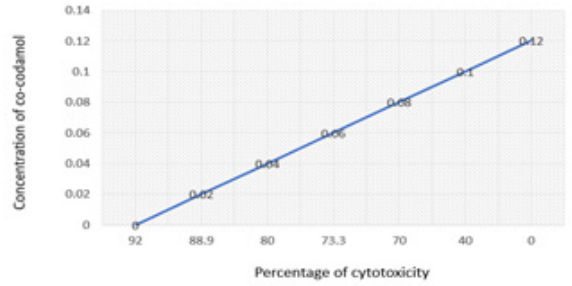

Fig. 2. Assessment of Co-codamol-Induced Cytotoxicity in Human Lymphocyte Cultures Using the MTT Assay. Percentage cytotoxicity of co-codamol at various concentrations (0.02–0.12 mg/mL) measured by the MTT assay, demonstrating a dose-dependent increase in cytotoxic effects.

this concentration was 0, and the cytotoxicity was 100 (Figure 2). The cytotoxicity of cells with different concentrations of co-codamol is calculated according to the formula mentioned in the Materials and Methods paragraph above.

The genetic study of the research samples showed chromosomal changes that included gap and normal group) for culturing lymphocytes at different concentrations. Chromosomal changes were recorded according to Table 2 and Figures 3 and 4. Compared to the normal group (not treated with co-codamol here, the concentration of the substance is zero). There was also a large spread of inhibition with a statistically significant decrease ($P < 0.05$) in the division index (4.7%) while in the normal it was (6.3%) and as in Table 2 and Figures 1 and 2, the mitotic index (MI), i.e. the frequency of metaphase nuclei, was measured against human lymphocyte cell lines using the mitotic index test. Evaluation of the genotoxicity of physical and chemical agents. The ace was given in the formula mentioned above in Materials and Methods. As for the micronucleus test, the percentage of micronuclei in diploid cells was 0.5% in normal groups. As for the group of samples after treatment with cocodamol concentration, there was a significant increase ($P < 0.05$) and the average frequencies of micronuclei in diploid lymphocytes by 1.9% compared to the healthy group. (Table 2)

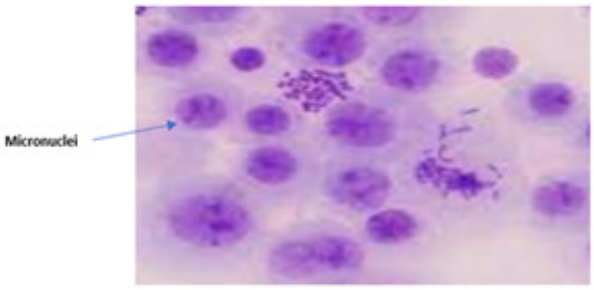

Fig. 3. Mitotic Index Analysis in Human Lymphocytes Treated with Co-codamol. Representative microscopic image (1000×) showing mitotic figures in human lymphocyte cultures after exposure to different concentrations of co-codamol, used to calculate the mitotic index.

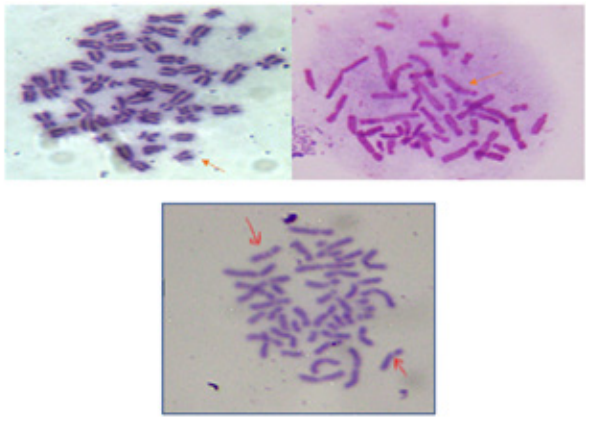

Fig. 4. Chromosomal Aberrations Observed in Human Lymphocytes Following Co-codamol Treatment. Representative microscopic image (1000×) illustrating structural chromosomal aberrations, including gaps and breaks, in lymphocytes exposed to increasing concentrations of co-codamol compared to control.

4. Discussion

Many studies and research have shown the cellular genetic effect of drugs on human cells [20,21]. The use of painkillers and medications is a major problem in the world and Iraq in particular, and since the spread of the Coronavirus, the use of a drug has become widespread. Research has been conducted on chromosomal changes and studies on drug addicts in Arab countries, especially after the recent epidemic. [22,23]. The results of the MTT test showed an inverse relationship with cell vitality. When

Table 2. Total micronucleus and mitotic chromosome index in human lymphocyte cultures.

Groups	Mitotic index	% Total chromosomal aberrations	% Micronuclei
Control	6.3	0.1	0.5
Conce.0.02*	6.1	0.1	0.6
Conce.0.04	5.8	0.2	1.0
Conce.0.06	5.7	0.3	1.3
Conce.0.08	5.5	0.5	1.5
Conce.0.1	5.0	0.7	1.7
Conce. 0.12	4.7**	0.9**	1.9**

the concentration of the drug increases, cell vitality decreases. MTT is an important test for measuring the toxicity of the mixture and its effectiveness in cell division. By measuring the activity of the purple enzyme MTT, which is mainly present in mitochondria and is active in high mitochondrial activity. This can be used to determine the cellular toxicity of potential drugs and other toxic substances [24,11]. Another issue is the future effect. This is a serious problem when toxicity is linked to genes and the occurrence of cancer [25,26]. Therefore, it is necessary to know the toxicity and duration of use of pharmaceutical compounds during their interaction with cellular molecules. The results in this area showed the following important points, a significant increase in the frequency of cells with chromosomal damage compared to the normal control group. An increase in chromosomal damage was found when using different concentrations of the drug. They studied the effects of the substances on genetic material by examining mitotic index (MI) and chromosome function when lymphocytes divide into human blood cells. This drug significantly increases cell division rates at concentrations (0.02 to 0.08) for approximately the same time (4, 12, and 72 hours). Co-codamol also has an inhibitory effect on MI at high concentrations (0.1-0.12). This effect is time-dependent. The toxic effect is an increased rate of chromosomal aberrations, especially in treated cultured cells, both of which are affected by time and concentration of the substance. Although we did not find any old or new sources dealing with the study of the genetic toxicity of this specific analgesic drug, this is the first study to identify changes or effects on the genetic material. To discuss the results, we found studies that included other analgesics or paracetamol alone or in combination with another analgesic. Among these cases, the rate of change was found to be much higher in cells (and micronuclei examined). by Karmakar et al. [27] treated at low (0.05, 0.1) and long-term (72 h) concentrations, indicating acentric chromosomal fragments or entire chromosomes. These are small chromatin-containing bodies that arise from nuclei that are attached to daughter nuclei after division. Therefore, a micronucleus test has been viewed as a biomarker of mitotic damage, leading to chromosome breakage or chromosome loss [28,29]. Some studies have concluded that this toxic effect may be related to the production of micronuclei through the presence of micronuclei in some cells of this drug user. This is consistent with [30,31,32]. Conclusions: Increasing the concentration of the drug decreased the viability of the cells. All cells were killed at the concentration of 0.12 mg/ml, which is the highest concentration. When MT was examined for measurement, the results showed that the cytotoxicity of the compound, cytotoxicity, and co-codamol concentration had Increased. From these results, it can be hypothesized that this combination of two analgesic drugs is used to treat severe diseases, can affect cell division and viability, and is genotoxic at high concentrations and repeated administration. This demonstrates the potential for genotoxicity in vivo and ex vivo, possibly through indirect cytotoxic effects or enzyme inhibition. These in vitro findings provide important evidence for the genotoxicity of the human cell culture system co-codamol.

This in vitro study provides compelling evidence that co-codamol exhibits genotoxic potential in cultured human lymphocytes. The observed decrease in cell viability, reduction in the mitotic index, and significant increase in chromosomal aberrations and micronucleus formation at higher concentrations of co-codamol strongly suggest that this commonly used analgesic can induce DNA damage and disrupt cellular division. These findings highlight the importance of cautious and judicious use of co-codamol, particularly at high doses or with prolonged administration. Further research is warranted to investigate the in vivo genotoxic effects of co-codamol and to elucidate the underlying mechanisms of its toxicity, ultimately informing clinical guidelines and minimizing potential risks associated with its use.

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