Original Research Article

Cytotoxicity and Genotoxicity of Sunset Yellow and Potassium Sorbate in Jurkat Cell Line

ABSTRACT

Aims: Industrial proceeded food, pharmaceutical and cosmetical products contain numerous substances whose safety have been widely concerned. Potassium sorbate (PS) is a common preservative used in a wide-range of products. Sunset yellow (SY) is one of the most commonly used dye in food and pharmaceutical industry. Several studies suggested cytotoxic and genotoxic potential of SY and PS in different cell lines. These effects can lead to organ damage and cancer development. The aim of this study was to investigate cytotoxic and genotoxic potential of two widely used food additives SY and PS in Jurkat cell line.
Methodology: PrestoBlue assay was used to assess the cytotoxic potential of SY and PS. For observing DNA damage, cytostatic and cytotoxic effects the cytokinesis-block micronucleus cytome (CBMN) assay was performed.
Results: Decrease of cell viability in Jurkat cell line was observed after 24h exposure to both SY and PS. CBMN assay has revealed significant increase of necrotic cells ($P<0.05$). Genotoxic biomarkers were in physiological range after 24h exposure to both analyzed additives.
Conclusion: Our findings suggest that SY, as well as PS have cytotoxic potential in Jurkat cell line, as a result of increased number of necrotic cells. Higher cytotoxic effect was caused by SY compared to PS. However, genotoxic potential was not recorded for any analyzed food additives.

Key words: cytotoxic effect, genotoxic effect, dye sunset yellow, preservative potassium sorbate, food additives.

1. INTRODUCTION

Food additives are often used in modern diet. They are usually classified into several functional classes including dyes, preservatives, sweeteners, acidity regulators, antioxidants, emulsifiers, stabilizers and thickeners [1]. An important issue, which concerns human health, is that the content of these additives is very often not stated on the packaging of the food products. In addition to the above, their quantity often exceeds the maximum permitted values leading consumers to ingest larger amounts of food additives than recommended by EFSA (European Food Safety Authority) [2].

Food dyes do not have any nutritional value. They are used in order to prevent color loss during technological processing or coloring of food, beverages, supplements and drugs[3]. Sunset yellow (SY), as one of the most common dyes used in food and pharmaceutical industry, is used with only one goal, which is to make the finale product more visually appealing and attractive [4]. It is a synthetic dye that belongs to the group of azo colors [5]. It is an orange water-soluble anionic monoazo dye [6]. The recommended acceptable daily intake (ADI) by EFSA is 4 mg/kg body weight per day for all population groups [7]. In a study performed in lymphocyte cell culture, changes in the mitotic index and micronucleus were observed after exposing the cells to SY, suggesting potential cytotoxicity and genotoxicity, in a dose-dependent manner [2]. Several studies have shown that SY can induce cytotoxic effects in human cell lines [2][8], which can lead to organ damage and various diseases. Recent study on chick embryos suggested SY as a possible nephrotoxic and hepatotoxic agent. In the SY treated group, liver and kidney necrosis, destruction of the renal cortex and cytoplasmic degeneration of hepatocytes were observed [9]. These findings raise concern about the need to reassess the no observed adverse effect level (NOAEL) for this particular additive.

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Potassium sorbate (PS) is one of the most commonly used additives, present in food, hygiene products and cosmetics. It serves as a preservative which prolongs the shelf life of food and prevent its spoilage caused by microorganisms. The ADI for PS is 3 mg/kg body weight per day [10]. Its toxicological profile regarding adverse effects on human health is the subject of a very few studies. In a study in isolated human lymphocytes, Mamur et al. showed that PS can cause an increase in chromosomal aberrations and disruptions of DNA strands [11]. Considering that genotoxic effects and chromosomal aberrations may be associated with the induction of carcinogenesis [12], it is of great interest to extend the research to other human cell lines, such as lymphocyte.

Lymphocytes are part of immune system which play an outsized role in defending organism from microorganisms, allergens and toxic chemicals. In our study Jurkat cell line was used as *in vitro* model. Jurkat cells are an immortalized line of human T lymphocyte cells.
Although there is an increased scientific interest in studying the cytotoxic and genotoxic effects of food additives, there is still lack of well-designed studies regarding their toxicity *in vitro* and *in vivo*. The objective of this study was to obtain preliminary information on the human toxicity of relatively frequently used additives as well to assess the cytotoxic and genotoxic effects of SY and PS in Jurkat cells.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents, and kits

All analyzes were performed using analytical grade chemicals and reagents. Double-distilled deionized water was used for the solution preparations and dilutions. PrestoBlue™ cell viability reagent was obtained from Thermo Fisher Scientific (Massachusetts, USA). Both chemicals, SY and PS, were obtained from Sigma-Aldrich® (St. Louis, Missouri, USA).

2.2. *In vitro* culture of Jurkat cell line

Cells were cultured in Gibco Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich®,St. Louis, Missouri, USA) supplemented with 10% Heat-Inactivated Fetal Bovine Serum (HI FBS) and 1% penicillin/streptomycin antibiotics (Sigma-Aldrich®, St. Louis, Missouri, USA), in humidified atmosphere containing 5% CO_2 at 37°C. For each experiment cells were grown to 80% confluence in 75 cm^2 culture flasks. All experiments were performed in triplicate in two independent experiments.

2.3. PrestoBlue cytotoxicity assay

For each experiment Jurkat cells were seeded (2 x 10^5 cells/well) in 24 well plates and grown for additional 24 h after exposure to SY and PS. Stock solutions and dilutions for SY and PS were prepared in double-distilled deionized water and solutions were sterilized by filtration through 0.2 µm sterile syringe filters. Afterwards, cells were treated with 10% SY or PS solutions and 90% RPMI culture medium in final concentrations of SY (0.125-1 mM) and PS (2.5-10 mM). Untreated cells were used as negative control, while positive control were cells treated with doxorubicin (20 mM) in RPMI nutrient medium.

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Do these concentrations correspond, for example, to the maximum concentration recommended by the EFSA? Or are they very high or low concentrations? This information needs to be clear as it will better guide the impacts of the results.

The PrestoBlue assay was carried out according to the manufacturer's instructions. After 24h of treatment with additives, cells were incubated with the reagent. The fluorescence was measured after 2h of incubation using spectrofluorophotometer RF-5301 PC (Shimadzu, Kyouto, Japan) at λex=530 nm and λem=582 nm. The measured fluorescence values were converted to percent of cell viability with respect to negative control.

2.4. Cytokinesis-block micronucleus cytome (CBMN) assay

The cytokinesis-block micronucleus cytome assay was used for observing DNA damage, cytostatic and cytotoxic effects. The mitochondrial cytochrome b (Cyt-B) gene was used to block cytokinesis. Solution of Cyt-B was added to treated cells to obtain final concentration of 4.5 µg/ml.After 28h incubation in CO_2 incubator cells were harvested and mounted on slides by cytocentrifugation. Then, slides were treated with methanol, May-Grünvald and Giemsa (MGG) stain [13]. Ultimately, slides were washed with deionized H_2O, dried on air, and examined under the microscope DM 1000 (Leica, Weltzar, Germany).

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2.5. Statistical analysis

Descriptive statistical analysis, as well as parametric One-way ANOVA test were carried out using SPSS software, version 21 (IBM, New York, USA). The results were considered statistically significant at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity assessment

To assess the cytotoxic potential of SY and PS, PrestoBlue viability assay was performed. PrestoBlue is a resazurin based reagent and highly sensitive assay for assessing cell viability and cytotoxicity [14]. Decrease of cell viability in Jurkat cell line was observed at four concentrations 0.125mM, 0.25mM, 0.5mM and 1mM of SY. Analyzed food dye had an effect on cell viability and caused dose-dependent cytotoxic effect (Figure 1).

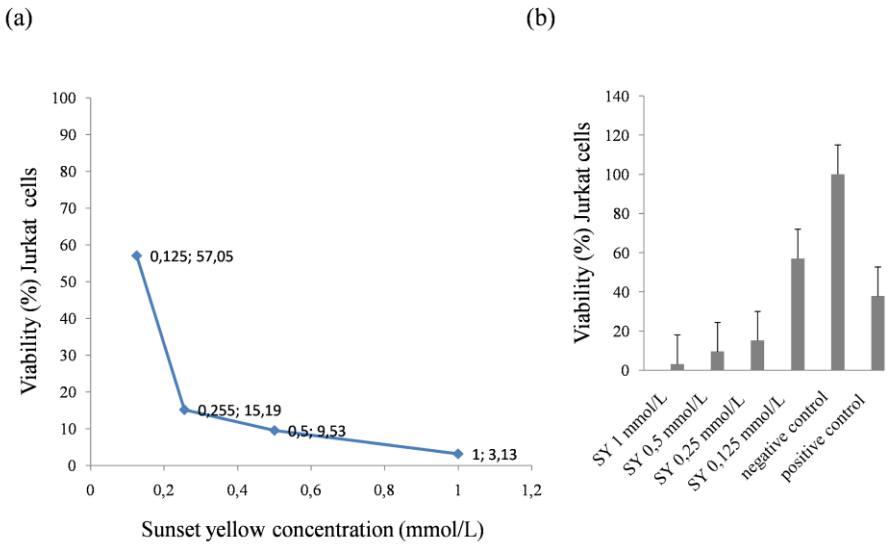

Figure 1. Viability of Jurkat cells after 24 hours of exposure to SY (a);(b).

Water solutions of PS were added to Jurkat cells, to obtain final concentrations of 2.5 mM, 5 mM, 7.5 mM and 10 mM. Decrease of cell viability was observed at three concentrations including 5 mM, 7.5 mM and 10 mM PS in Jurkat cell line after 24h of exposure (Figure 2). Compared to cytotoxic effects of SY, PS showed lower cytotoxic effects in Jurkat cells.

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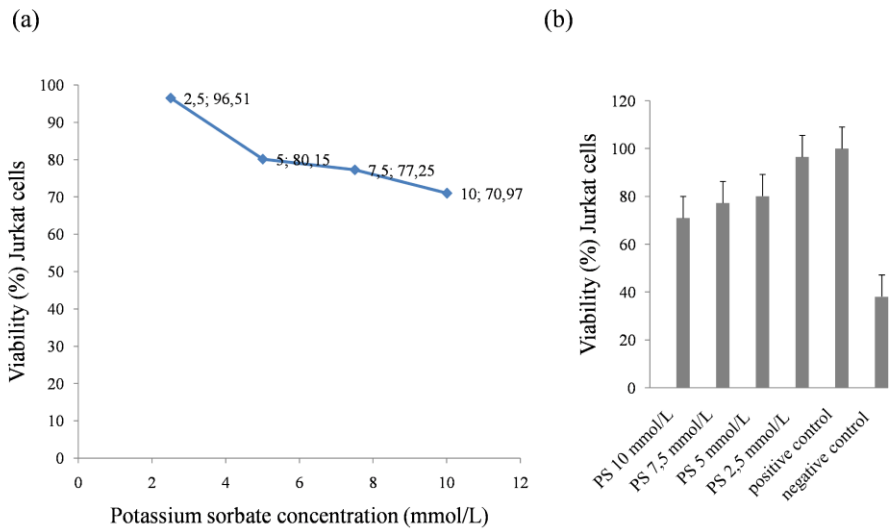

Figure 2. Viability of Jurkat cells after 24 hours of exposure to PS (a); (b).

As shown in Figures 1 and 2 concentration of 1 mM SY has shown the greatest cytotoxic effect, observed in 96.87% of treated cells.On the other hand, the highest concentration of PS caused the greatest cytotoxic effect, observed in 29.03% of treated cells.From the obtained results it can be seen that the cytotoxic effect of the lowest concentration SY solution (0.125 mM) was as much as 1.48 times greater than the cytotoxic effect of the highest concentration PS solution (10 mM).Therefore, it is reasonable to assume that compared to the cytotoxicity of SY, PS shows less cytotoxic effect in Jurkat cells.

3.2. Genotoxicity of SY and PS

Cytokinesis-block micronucleus cytome assay was used to assess DNA damage, cytostasis and cytotoxicity. Nuclear division index (NDI), percentage of apoptotic and necrotic cells, frequency of binuclear (BN) and multinuclear cells, cells with micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored and calculated (Figure 3).

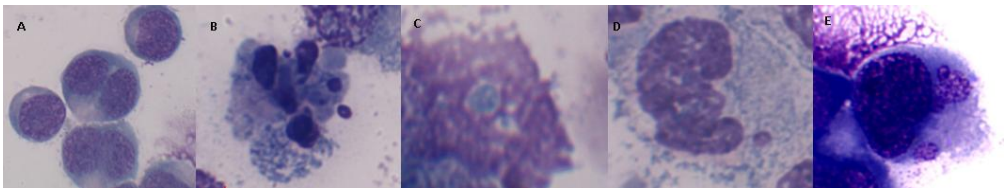

Figure 3. CBMN assay: A) mononuclear and binuclear cells; B) apoptotic cell; C) necrotic cell; D); cell with MNi; E) cell with NPB and MNi

For nutrition studies in human lymphocytes, NDI is a marker of cytostasis and immune response, determining a mitogen response. In order to obtain NDI, a total of 500 viable cells were scored to determine the frequency of cells with 1- 4 nuclei. NDI was calculated using following equation:

$$\mathrm{NDI} = \frac{\mathrm{M_1} + \mathrm{M_2} + \mathrm{M_3} + \mathrm{M_4}}{\mathrm{N}}$$

where M_1-M_4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored [15].

The obtained results indicated that NDI values were within the physiological range values (Table 1). Cells with genotoxicity biomarkers NPBs, MNi and NBUDs were rare, and their numbers were in the physiological range, as well. Although the number of apoptotic cells, when treated with 1mM SY, was significantly different compared to the negative control, according to Fenech obtained result was alsoin the physiological range [15]. However, the number of necrotic cells was increased after exposure to SY (*P*<0.05). Accordingly, the number of cells with genotoxicity biomarkers NPBs, MNi, NBUDs, and NDI values were in the physiological range (Table 1).

Table 1. Genotoxicity of SY using CBMN assay

SY (mM)	Number of cells					
	NDI	Necrotic cells	Apoptotic cells	NPBs per BN 1000 cells	MNi per 1000 BN cells	NBUDs per 1000 BN cells
Negative control	1.58±0.08	0.00	0.76±0.22	0.00	0.00	0.00
1	1.32±0.01	28.27±0.86*	4.07±0.45*	0.00	1.00±0.00	0.00
0.50	1.44±0.28	19.84±0.61*	1.04±0.52	1.50±0.71	1.50±0.71	0.00

*Significantly increased against negative control (*P*<0.05).

Obtained results with CBMN assay are consistent with our results for cytotoxicity with PrestoBlue assay, and showed that necrosis was the cause of decrease in cell viability. The results showed dose-dependent effect (Table 1).

Similar to SY, PS showed significant increase in the number of necrotic cells (*P*<0.05). These results confirmed our hypothesis that cell necrosis was the main cause for decrease in cell viability after exposure to PS. The percentage of apoptotic cells, NDI and genotoxic biomarkers were within the physiological range (Table 2), suggesting that PS have no genotoxic potential.

Table 2. Genotoxicity of PS using CBMN assay

PS (mM)	Number of cells					
	NDI	Necrotic cells	Apoptotic cells	NPBs per BN 1000 cells	MNi per 1000 BN cells	NBUDs per 1000 BN cells
Negative	1.58±0.78	0.00	0.76±0.22	0.00	0.00	0.00

control						
10	1.28±0.35	22.94±1.04*	3.97±0.23	2.00±1.41	4.00±0.00	0.00
5	1.45±0.28	18.32±1.47*	2.62±0.02	0.00	0.50±0.71	0.00

*Significantly increased against negative control (P<0.05).

The results obtained in this study are consistent with the results reported in other similar *in vitro* studies on cytotoxic and genotoxic effects of additives [8][11][16][17]. We have confirmed that both additives, SY and PS, caused decrease in cell viability in Jurkat cell line (Figure 1 and 3). As decrease in cell viability is inversely proportional to cytotoxicity, we found that SY, in all concentrations used, have caused dose-dependent increase of cytotoxicity in Jurkat cells compared to the untreated cells. Further, CBMN assay confirmed our results obtained by using PrestoBlue assay and revealed the type of cell death after exposure of Jurkat cell line to SY. The number of necrotic cells was significantly increased in dose-dependent manner for two analyzed concentrations of SY (0.5 mM and 1 mM). Necrosis is a type of uncontrolled cell death, which can occur as a result of the toxic effects of heterogeneous chemicals[18]. Obtained results are in accordance with Kus and Eroglu, which suggested cytotoxic potential of SY in human lymphocytes [2]. Similar effects to those of SY have been recorded in Jurkat cell line caused by PS. Our results are comparable with the results published by Qu et al, which suggest cytotoxic potential of PS [16].

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NDI is a marker of cell proliferation and provides an estimation of mitogen response [15]. Although NDI values were in the physiological range for cells treated with SY or PS, we observed decrease in NDI value with increase of SY and PS concentration. Furthermore, obtained NDI values for SY and PS treated cells are lower than NDI for untreated cells. Since the number of cells with genotoxic biomarkers were in the physiological range for SY and PS, we can assume that analyzed food additives have no genotoxic potential, which is also previously reported by Özdemir et al. and Sasaki et al.[17][19]. On the contrary, Dehghan et al. in their review reported PS as both, cytotoxic and genotoxic agent, when humans are exposed to it in a concentration greater than 25 mg/kg [20]. Therefore, additional studies on the toxicity of additives have significant importance.

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Food additives such as a potassium salt of sorbic acid PS used as an effective preservative and antimicrobial agent, and food coloring agents such as azo-dye SY are very popular additives with applications in food, cosmetic and pharmaceutical industries due to their low cost, good stability and effectiveness. It is not unusual to use these additives together in the same product. Despite their good properties and advantageous industrial use, there is a rising concern regarding associated health risks due to the exposure to these substances. It is known that they can be the cause of adverse health effects including respiratory, neurological and gastrointestinal problems, allergies, infertilities, eczema, etc. while some recent studies pointed out to their ability to cause cytotoxic and genotoxic effects, as well as to induce carcinogenesis [20][21][22]. These products are usually used at very low concentrations. However, they are often preferred together and with other chemicals with similar mode of action in food products, medicines, cosmetics, etc. increasing the occurrence and the human health risk. Chemicals with the same target organ of toxicity very often act in an additive manner. Therefore, integrative approach in a risk assessment could lead to a much higher health risk than each chemical compound would exhibit individually [23]. As we previously showed, two chemicals can act independently from each other, or they can interact whereby the effect of the toxic mixture may result in additive, synergistic, potentiating or even antagonistic effect [24]. Accordingly, the combined effects of SY and PS should be also considered in the future research.

4. CONCLUSION

The results obtained in this study suggest that both frequently used food and pharmaceutical additives, SY and PS, have significant cytotoxic effect on Jurkat cells. Nevertheless, higher cytotoxicity was shown by SY compared to PS. In addition, the results obtained by this study indicate that cell necrosis is the main cause for decrease in cell viability after exposure to SY and PS.

In the terms of the genotoxicity, both analyzed additives did not show increased genotoxicity compared to the untreated cells. These findings suggest that SY and PS are not genotoxic agents on Jurkat cells.

In order to evaluate cytotoxicity and genotoxicity of these two additives individually and in a chemical mixture more accurately, more studies on other human cell lines are needed.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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