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Determination of the cyto-genotoxic effects of sodium acetate and sodium sulfite by MTT and comet assays

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ABSTRACT

Sodium acetate (NaA) and sodium sulfite (NaS) are two food additives in the class of preservatives. In this study, 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT) assay was established to detect the cytotoxicity, and comet assay was used to determine the genotoxicity of NaA and NaS. For the MTT assay, human hepatocellular carcinoma ($HepG_2$) cells were treated with different concentrations of each preservative (15.63, 31.25, 62.50, 125, 250, 500, 1000 and 2000 µg/mL for NaA; 3.91, 7.81, 15.62, 31.25, 62.50, 125, 250 and 500 µg/mL for NaS, respectively) for 24-h. non-treated wells used as control (only medium) were included. Comet assay was performed on lymphocytes isolated from healthy donors with multiple concentrations of NaA (15.63, 31.25, 62.50, 125, 250 µg/mL) and NaS (3.91, 7.81, 15.62, 31.25, 62.50 µg/mL) for 1 h. A negative (distilled water) and a positive control (100 µM H_2O_2) were also included. Significant cytotoxic activity was detected for NaA and NaS only at the highest concentration. Besides, both substances significantly increased DNA damage compared to the control at almost all concentrations (except at low concentrations). In general, both food preservatives exhibited weak cytotoxic effects in $HepG_2$ cells. These food preservatives showed genotoxic activity, especially at higher concentrations.

Keywords: Comet assay, Food preservative, MTT assay, Sodium acetate, Sodium sulfite

Introduction

Food additives are substances added to foodstuffs to prevent and/or delay product spoilage and to improve or modify its properties, such as flavour and appearance. These substances can be natural or synthetic, ordinarily have no significant nutritional value, and are added to food in specific quantities during production. According to their functions, 25 categories of food additives have been defined. Food preservatives, also called antimicrobial agents, are utilised to prolong the shelf life of food by safeguarding it against spoilage caused by microorganisms, and their use is EU-regulated (Silva & Lidon, 2016; Zang et al.,2023). Microbial spoilage is the gradual decrease in food safety due to the movement of different types of bacteria. Components such as carbohydrates, proteins, vitamins, minerals, and water in foods create a suitable area for bacteria to grow. The oxidation-reduction potential, water activity, and pH value of the food are also significant. The essential foods affected by microbial spoilage are vegetables, fruits, fruit juice, milk, dairy products, cereals and cereal products, and meats (Altuğ, 2009; Yen et al., 2024).

Sodium acetate (NaA, E 262) and sodium sulfite (NaS, E221) are two different food preservatives; NaA is the sodium salt of acetic acid, and NaS is the sodium salt of sulfuric acid (Sallam, 2007). NaA is used effectively in meat, cheese, bakery, snacks, cosmetics, and veterinary chemicals and herbicides. It prevents rotting by reducing the growth of bacteria in products (Mohammadzadeh-Aghdash et al., 2018). NaS preserves food by inhibiting yeast, fungal, and bacterial growth. It is a preservative in products containing egg yolks and foods like beer, salads, caramel, and bread. In addition to its preservative function, it can also be an antioxidant (Silva & Lidon, 2016).

Today, because of the rapid increase in the consumption of chemicals in all fields, it has become essential to determine whether they have a negative effect in terms of toxicology on human health. Cytotoxicity assays are a quick way to assess the impact of a particular chemical compound on a specific human cell line. The most widely used method today is the MTT assay. This method originated as a cell proliferation assay and was used in the same years to investigate the effect of chemotherapeutic drugs on cancer cells (Mamur, 2022a; Ghorbanpour et al., 2024). The MTT assay is based on the conversion of yellow 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a water-insoluble purple colour in living cells due to mitochondrial dehydrogenase enzyme. The intensity of the colour is directly related to mitochondrial activity. If there is activity in living cells, formazan crystals are formed. In dead cells, formazan crystals cannot be formed. In summary, the yellow colour of MTT dye turns purple with the addition of solvent due to living activity. This colour change is then read in the microplate reader, and data is obtained using optical density (Mossman,1983). The MTT assay is reliable for measuring cell viability, activation, and growth and is widely used in cytotoxicity studies, which determines the percentage of living cells (Ghasemi et al., 2021).

Genotoxicity tests are performed to determine whether chemical and physical agents cause chromosome abnormalities, mutations, and DNA damage and to understand the mechanisms of action of these agents. Various *in vivo* and *in vitro* test systems are used for this purpose (Unal et al., 2021; Mahoro et al., 2024). The comet assay method is preferred in research studies to determine DNA damage and repair disorders induced by different chemical and physical agents in multiple mammalian cells. It is a preferred biomonitoring method for detecting increased DNA damage in some diseases (such as cancer). On the other hand, it has a widespread framework of use due to its advantages, such as its ability to evaluate genotoxic substances first in their sites of action, its application in almost all eukaryotic cells, its ability to measure low levels of damage, and the fact that it is a fast, practical, simple and convenient method because it requires very few cell samples. This assay quantitatively detects DNA damage (Collins et al., 2014; Costea et al., 2024). In the comet assay, three parameters were used to measure DNA damage levels: tail intensity, length, and tail moment. Enhanced comet tail intensity and tail moment are directly related to increased DNA damage. The most commonly used comet parameters are tail intensity (%DNA in tail) and tail moment, which integrates %DNA in tail and tail length (Azqueta et al., 2019). Comet tail intensity is considered the most helpful comet parameter since it is not affected by experimental conditions and can measure the broadest range of DNA damage (Collins et al., 2014).

Many food additives are categorised as Generally Recognized as Safe (GRAS) in terms of potential risk, including NaS and NaA (EFSA, 2022a, b). However, some studies in the literature showed that these additives may possess a cytogenotoxic risk. For example, Sun et al. (2005) found that NaA at 50-100 mM (24-72 h) caused reduced viability in human gastric adenocarcinoma epithelial cells. In another study, NaA (12.5, 25-, and 50-mM for 24 h) increased the release of LDH, an indicator of cytotoxicity and cell death, in the human gastric adenocarcinoma epithelial cell line (Xia et al., 2016). Similarly, NaA was cytotoxic and genotoxic *in vitro* for lym-

phocytes isolated from adult male Sprague-Dawley; it increased DNA damage percentage, increased LDH release, decreased cell viability and proliferation of lymphocytes at all concentrations (50, 100, and 200 mM/L) (Abd-Elhakim et al., 2018). Mohammadzadeh-Aghdash et al. (2018) indicated that NaA reduced HUVEC cell line growth in a concentration (25, 50, 100, and 200 μM) and time-dependent manner (24 and 48 h). In addition, sulfur dioxide derivatives (125, 250, or 500 mg/kg body wt, intraperitonally for 7 days), including NaS and sodium bisulfite (3:1 M/M), exhibited significant increases in DNA damage in all tested organs (brain, lung, heart, liver, stomach, spleen, thymus, bone marrow and kidney) in male mice (Meng et al., 2004). El-Hefny et al. (2020) demonstrated that NaS (100 mM) has significantly high tumour induction and frequency levels in the SMART assay and a large amount of DNA damage in the comet assay. Finally, in a study conducted in 2023, it was found that NaA (15.63-250μg/mL) and NaS (3.91-62.50 μg/mL) increased the frequency of chromosomal aberrations (CAs) and micronuclei (MN) at higher concentrations (Altunkaynak, 2023).

This study aimed to measure the cytotoxic effects and primary DNA damage capacity of sodium acetate (NaA) and sodium sulfite (NaS). For this purpose, MTT assay in hepatocellular carcinoma ($HepG_2$) cell line, since they are of human origin and retain xenobiotic metabolising enzymes which play a crucial role in the activation/detoxification of genotoxic chemicals (Knasmüller et al., 1998) and comet assay in isolated human lymphocytes, was performed for the first time using various concentrations of NaA and NaS.

Materials and Methods

Concentrations

In this study, MTT assay was used to determine the cytotoxic effect, and comet assay was used to evaluate the genotoxicity of NaA and NaS. The best suitable concentrations were determined using LD_{50} value [NaA: mouse orally 4960 mg/kg (PubChem, 2023) and NaS: rat orally 3560 mg/kg (Sigma-Aldrich, 2023)]. Based on the LD value (mouse orally 4960 mg/kg /4 = 1240 mg/kg ~ 2000 μg/mL) and MTT result for NaA, the highest concentration was chosen as 2000 μg/mL. LD value for NaS (rat orally 3560 mg/kg / 4 = 445 mg/kg ~ 500 μg/mL) was also considered. For the MTT assay, $HepG_2$ cells were treated with eight different concentrations of each preservative (15.63, 31.25, 62.50, 125, 250, 500, 1000 and 2000 μg/mL for NaA; 3.91, 7.81, 15.62, 31.25, 62.50, 125, 250 and 500 μg/mL for NaS) for 24 hours. Additionally, $HepG_2$ cells without any chemical treatment were used as the control group. MTT test results and the previous values (Altunkaynak, 2023) were also used to determine comet assay concentrations. In the comet assay, lymphocytes isolated from blood samples obtained from two healthy donors were treated with NaA concentrations of 15.63, 31.25, 62.50, 125, 250 μg/mL and NaS concentrations of 3.91, 7.81, 15.62, 31.25, 62.50 μg/mL for 1 hour. Distilled water was used as a negative control, and 100 μM H_2O_2 was maintained as a positive control.

$HepG_2$ Cell Line Cultures

The human hepatocellular carcinoma ($HepG_2$) cell line (HB-8065) used in experiments was commercially obtained from the American Type Culture Collection (ATTC, Manassas, USA). Human $HepG_2$ cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine in $75cm_2$ culture flasks under conditions (humidified, 5% CO_2/95% air, 37°C) to obtain the desired count. Cells that reached sufficient numbers two days after being placed in the culture flask were collected by trypsinisation. Subsequently, the cells were transferred to 96 multiwell plates.

MTT Assay

The cytotoxic capability of NaA (CAS. No: 127-09-3, Sigma-Aldrich) and NaS (CAS. No: 7757-83-7, Sigma-Aldrich) were evaluated using MTT assay in $HepG_2$ cells. The MTT assay determining the mitochondrial activity was applied according to Mossman (1983) with some modifications followed by Mamur et al. (2022b) methods. $HepG_2$ cells were grown in 96-well tissue culture plates at a density of 5×10^3 cells per well. Afterwards, the cells were incubated for 24 hours in a CO_2 incubator for the holding wells. Varying concentrations of NaS (3.91-500 μg/mL) and NaA (15.63-2000 μg/mL) were added to each well and incubated for 24 hours. Non-treated cells were used as the negative control (only medium). To complete the incubation, 5 mg/mL MTT solution was added to each well and incubated for 4 hours. A marker of cell viability is the conversion of the tetrazolium salt MTT to a coloured formazan by mitochondrial dehydrogenases. Dimethyl sulfoxide was added to each well to solubilise formazan crystals. Then, the absorbance (Abs) was measured at 570 nm wavelength using a spectrophotometer Molecular Devices M_5 microplate reader. The assay was performed in triplicate. The percentage of cell viability for each concentration and the IC_{50} value were then detected.

Comet Assay

The comet assay applied is based on the technique of Singh et al. (1988) with some amendments. Human peripheral blood samples were taken from two healthy donors, one male and

one female, aged between 22 and 27 years. None of the donors had problems that may have prevented them from being volunteers, such as health, alcohol, cigarettes, and drugs. This study was conducted with permission numbered 2023/121 obtained from the Amasya University Ethics Committee. Lymphocytes (separated from whole blood by biocoll) viability was quantified to be above 96% by the Trypan Blue Exclusion Test. Then lymphocytes were exposed to five concentrations of NaA (15.63, 31.25, 62.50, 125, and 250) and NaS (3.91, 7.81, 15.63, 31.25, and 62.50) and incubated at 37 °C for 1 h. A control (sterile distilled water) and positive control (H_2O_2, 100 µM) were also run. After time off the incubation, the cells were centrifugated at 1348 g, 5 min, resuspended using PBS, and were gently mixed with low melting agarose (0.65%). Next, the suspension was rapidly spread onto slides previously coated with normal melting agarose (0.65%), and coverslips were placed over the slides. The slides were put into a digestion or lysing solution for at least 1 hour at 4°C. Then, the slides were incubated in an electrophoresis solution for 20 min. The electrophoresis was performed at 25 V and 300 mA for 20 minutes. Slides were stained with EtBr (20 µg/mL) after washing in a neutralisation buffer. To determine DNA damage, a total of 200 cells for each concentration (100 cell/donor) were imaged through a dedicated Analysis System called Comet Assay IV (Perceptive Instruments Ltd., UK), with regards to three comet parameters (tail intensity (%), tail length (µm) and tail moment).

Data Analysis

The MTT cell viability assay was analysed using one-way ANOVA followed by Dunnet's multiple comparison tests. Statistical significance was defined as a p-value less than 0.05. To explore statistical significance, a t-test was performed on all comet parameters. A regression analysis was also performed to reveal the dose-response relation. IBM SPSS 22.0 software was used for statistical analysis.

Results and Discussion

Table 1 summarises the results of the MTT assay in $HepG_2$ cells treated with NaA and NaS, respectively. None of the concentrations of either food additive significantly affected the cell viability except the highest concentration. NaA exhibited a significant decrease in cell viability at the highest concentration (2000 µg/mL). Additionally, this concentration was determined as the IC_{50} value (cell viability is 51.57 %) that kills 50% of the population of the cells (Table 1, Figure 1). NaS showed a significant reduction in cell viability only at 500 µg/mL (Figure 2). The cell viability value at 500 µg/mL was 38 %. It was determined that NaA (2000 µg/mL) and NaS (500 µg/mL) showed low mitochondrial activity at the highest concentrations. On the other hand, NaA and NaS did not show cytotoxic effects in $HepG_2$ cells at all concentrations except at the highest concentrations applied.

The effect of NaA and NaS on three comet assay parameters is given in Tables 2 and 3, respectively. At all concentrations except the lowest (15.63 µg/mL), NaA significantly increased the comet parameters compared to the control. These increases were dose-dependent, but the correlation was weak for tail moment ($r = 0.48$) and tail length ($r = 0.44$). The correlation was relatively strong for tail intensity ($r = 0.75$). Similarly, NaS induced significant comet tail intensity at all concentrations except at 3.91 µg/mL (lowest concentration); however, the concentration of 3.91 µg/mL was also statistically significant for the other two comet parameters, tail length and moment, like as being other all concentrations. Also, it was found that a robust dose-dependent correlation between DNA damage induction and concentration for tail intensity ($r = 0.76$) and tail moment ($r = 0.89$), while tail length ($r = 0.41$) showed a weak dose-dependent correlation.

In this study, the possible cytotoxic effect caused by NaA and NaS was evaluated in $HepG_2$ cells by MTT assay, and their potential for damaging DNA was assessed in human lymphocytes using a comet assay. As a result, both food preservatives induced cytotoxic effects at high concentrations. However, at concentrations where DNA damage was observed in human lymphocytes, no cytotoxic effect was determined in $HepG_2$ cells. This result may be due to the difference in the cell group. Notably, a cyto-genotoxic effect with increasing concentration was observed for both test systems.

Table 1. Cytotoxic effect of sodium sulfite and sodium acetate in $HepG_2$ cell line

Test substance	Concentration (µg/mL)	N	Cell viability (24 h) Mean±SD
Control	0.00	3	3.014±0.111
NaA	15.63	3	3.242±0.067
	31.25	3	3.224±0.068
	62.50	3	2.918±0.387
	125	3	3.269±0.265
	250	3	3.195±0.273
	500	3	3.132±0.067
	1000	3	2.973±0.083
	2000	3	1.762±0.566 * #
Control	0.00	3	2.747± 0.163
NaS	3.91	3	2.889±0.084
	7.81	3	2.909±0.230
	15.63	3	3.023±0.247
	31.25	3	3.152±0.036
	62.50	3	2.375±0.090
	125	3	2.909±0.051
	250	3	2.534±0.028
	500	3	1.122±0.540 *

Non-treated cells were used as control. NaA: Sodium acetate; NaS: Sodium sulfite; $HepG_2$: Human Hepatocellular Carcinoma Cell Line; SD: Standard Deviation
* $p<0.05$ statistically significant according to the one-way ANOVA-Dunnet Test)
# IC_{50} value

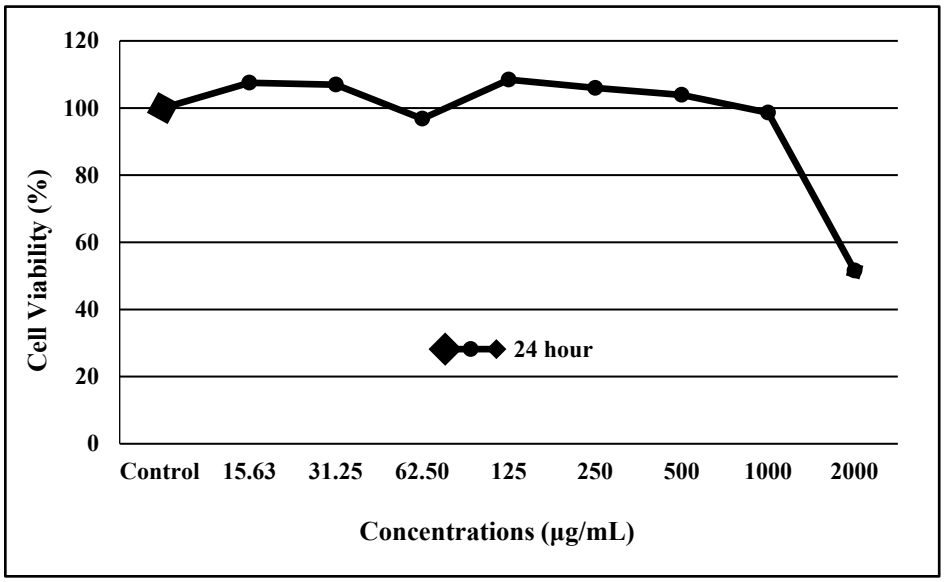

Figure 1. Cell viability result of sodium acetate in $HepG_2$ cell

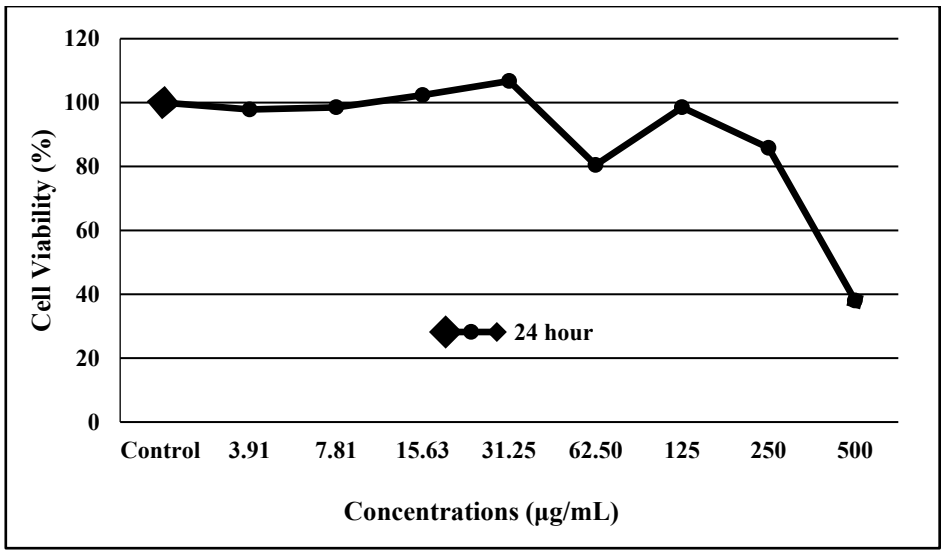

Figure 2. Cell viability result of sodium sulfite in $HepG_2$ cell

Table 2. Levels of DNA damage measured in isolated peripheral lymphocytes that were exposed to different concentrations of sodium acetate (NaA)

Test substance	Exposure time (hour)	Concentration (µg/mL)	Tail intensity (%)	Tail Lenght (µm)	Tail Moment
Control	1	0.00	18.23±1.38	65.91±1.35	4.94±0.40
Positive control (H_2O_2)	1	100 µM	28.82±1.88	198.79±9.20	18.76±2.15
NaA	1	15.63	17.67±1.38	67.96±1.71	5.15±0.59
		31.25	23.21±1.80*	102.08±4.56*	8.65±0.96*
		62.50	22.92±1.57*	87.87±3.20*	7.85±1.03*
		125	22.50±1.27*	79.32±2.65*	6.15±0.45*
		250	22.36±1.44*	83.96±3.00*	7.21±0.81*

* Significantly different from the control P< 0.05 (t-test)

Table 3. Levels of DNA damage measured in isolated peripheral lymphocytes that were exposed to different concentrations of sodium sulfite (NaS)

Test substance	Exposure time (hour)	Concentration (µg/mL)	Tail intensity (%)	Tail Lenght (µm)	Tail Moment
Control	1	0.00	17.30±1.37	69.76±1.39	4.69±0.43
Positive control (H_2O_2)	1	100 µM	28.57±1.97	156.16±9.56	20.41±2.75
NaS	1	3.91	20.10±1.75	88.64±2.53*	6.50±0.76*
		7.81	22.95±1.73*	94.10±3.45*	8.39±1.05*
		15.63	24.74±1.90*	85.31±2.46*	9.05±1.06*
		31.25	22.03±1.79*	79.43±2.56*	8.15±1.09*
		62.50	23.30±1.99*	90.63±2.85*	9.70±1.28*

* Significantly different from the control P< 0.05 (t-test)

The potential risks of food additives are regularly reassessed by the European Food Safety Authority (EFSA, 2022a,b). However, many food additives are still categorised as Generally Recognized as Safe (GRAS) and have not been assessed by the regulatory authorities. Much data is based on toxicity tests and assessments commissioned by manufacturers (Maffini et al., 2017; Neltner et al., 2013; Yen et al., 2024). Although both preservatives are Generally Recognized as Safe (GRAS) by EFSA (except for NaS in meats or food recognised as the source of vitamin B1), genotoxic and cytotoxic effects in some cell lines have been reported in the literature. Xia et al. (2016) investigated the NaA on the viability of the human gastric adenocarcinoma epithelial cell line using an LDH release assay. They reported that 6.25 mM NaA for 24 h exhibited a rise in LDH level, but the LDH releasing was fewer than 3%. Moreover, LDH release increased to 2.1, 3.8, and 7.0% after the cells were exposed to 12.5, 25-, and 50-mM NaA for 24 h. The authors concluded that NaA exerted an apoptotic effect in the human gastric adenocarcinoma epithelial cells via a caspase-dependent apoptotic pathway. The results presented in this paper showed that NaA was cytotoxic at higher concentrations, and this effect became more pronounced as the concentration increased. Since the LDH assay is also an indicator of cytotoxicity, the results were similar even though the concentrations used were different. Sun et al. (2005) determined that the lowest concentration of NaA (12.5mM) had increased viability after both treatment periods, 24 and 48 hours. The concentration of 25 mM NaA had almost no effect on the viability of human gastric adenocarcinoma epithelial cells. However, NaA at 50-100 mM caused reduced viability. These results stated that high concentrations of NaA in food have cytotoxic effects (Sun et al., 2005). Similarly, in this study using $HepG_2$ cells, some low NaA concentrations caused an increase in cell viability compared to the control group. Although not significantly reduced, cell viability decreased as the concentrations increased. Indeed, cell viability decreased after 62.50 µg/mL sodium acetate treatment (Figure 1), increased at 125, 250, 500, and 1000 µg/mL concentrations and suddenly decreased at the highest concentration (2000 µg/mL). Similarly, the cell viability, which decreased after 62.50 µg/mL of sodium sulfite treatment (Figure 2), increased with 125 µg/mL treatment and decreased following high concentrations. Therefore, the 62.50 µg/mL concentration may be interpreted as the threshold concentration known as the lowest dose at which the induced effect occurs within the framework of the dose-response relationship. However, these increases and decreases were not statistically significant compared to the control. In addition, as the concentration increases, the observed changes seem small. Meng et al. (2004) evaluated the DNA damaging effects of a mixture containing NaS and sodium bisulfite (3:1 M/M; sulfur dioxide derivatives) on various organs of male mice. Sulfur dioxide (SO_2) derivatives received an intraperitoneal (125, 250, 500 mg/kg body weight) daily for a week. Their results demonstrated that SO_2 and its derivatives significantly increased DNA damage (olive tail moment) in all organs tested from male mice, providing systemic toxicity. Abd-Elhakim et al. (2018) found that NaA increased DNA damage percentage on lymphocytes isolated from adult male Sprague-Dawley rats; tail DNA percentage, length, and moment in a dose-dependent manner at all tested concentrations (50, 100, and 200 mM/L). Additionally, all NaA concentrations decreased cell viability (MTT assay, exposure time: 24 h and 72 h) and proliferation (72 h) of lymphocytes and increased LDH release, concentration-dependently. After all, NaA was suggested to be cytotoxic and genotoxic for lymphocytes isolated *in vitro*. El-Hefny et al. (2020) investigated the DNA-damaging effect of NaS using the SMART and comet assay. In the SMART assay, two different *Drosophila* strains were used, a wild-type strain and a strain carrying *wtsMT*$^{4-1}$ (a lethal wart allele balanced on TM3), and larvae were exposed to NaS (100 mM) for approximately 48 hours. NaS significantly increased the frequency of *warty* tumours in *D. melanogaster* flies. Furthermore, the comet assay evaluated all cell types of the isogenic w^{1118} strain of *D. melanogaster* exposed to NaS (100 mM, 24 h) for DNA breaks. NaS caused significant DNA damage. The researchers commented that NaS has a noticeable genotoxic potential. Remarkably, NaS also appears to cause DNA damage in *in vivo* conditions. In the present study, NaA and NaS increased the comet tail intensity, length, and moment in isolated lymphocytes at almost all concentrations. Both NaA and NaS significantly caused DNA damage. In this respect, the data obtained from the two previous studies are consistent with the present study. Besides, Mohammadzadeh-Aghdash et al. (2018) exhibited that NaA (25, 50, 100, and 200 µM) decreased HUVEC cell line growth in a dose and time-dependent manner (IC_{50} value: 487.71 µM at 24 h and 232.05 µM at 48 h); however, it did not induce a significant effect on DNA fragmentation or smear upon exposure of HUVECs with IC_{50} concentration of NaA using DAPI staining and DNA ladder assays. Moreover, using FITC-labeled Annexin V flow cytometry, IC_{50} concentration of NaA did not lead to considerable apoptosis or necrosis. They concluded that a low concentration of NaA did not have significant cytotoxic and genotoxic effects. On the other hand, the absence of DNA damage and increased cell death in cells exposed to IC_{50} concentration may be due to the difference in the cells used in the experimental sets. Therefore, it is clear that the effects of both NaA and NaS should

be investigated in different cell groups. In a study conducted in 2023, the genotoxic effects of NaA and NaS were examined in human lymphocytes by chromosomal aberration and micronucleus (MN) tests. For this, 15.63-250 µg/mL of NaA and 3.91-62.50 µg/mL of NaS were used. Both food preservatives increased the frequency of CAs and MN at higher concentrations. In the same study, mitotic index values also determined the cytotoxicity of NaA and NaS at the same concentrations in human lymphocytes. The mitotic index, an indicator of cytotoxicity, decreased statistically significantly at the high concentrations of NaA and NaS used in cultured human lymphocytes. In summary, NaA and NaS showed both genotoxic and cytotoxic effects at high concentrations in human lymphocytes (Altunkaynak, 2023). The study's results are consistent with this study regarding both aspects (cytotoxicity and genotoxicity).

According to the recent EFSA report (2022a), sulfites, including NaS, may be unsafe, especially at high concentrations. Ishidate et al., 1984, reported no mutagenicity in the Ames assay and no induction of chromosomal abnormalities in mammalian cells in the *in vitro* chromosomal aberration test. However, in later years, a mutagenic effect was observed in the spore rec test with the *Bacillus subtilis* M45 strain (Ueno et al., 2002). Additionally, it caused gene mutation at the gpt locus of AS52 cells (Meng & Zhang, 1999), which was associated with a cytotoxic effect at the highest concentration used. In this study, the damage at high concentrations was observed more prominently, so the cytotoxic effect observed at high concentrations may be related to DNA damage. Together with the results of the other studies described above, the EFSA report states that there are gaps in the toxicity data of NaS and other sulfites and that the effectivity of this situation on health still needs to be determined. Another report by the same authority announces that acetates (including NaA) used as food additives should be re-evaluated (EFSA, 2022 a, b). Considering the studies conducted, the increase in mutagenicity and genotoxicity, especially at high concentrations, and the finding of different results in different cell groups have caused concern. An example is the finding of completely different mutagenicity results in different prokaryotic organisms in the studies described above. Taken together, as stated in EFSA's recent reports on sulfites and acetates, there is a significant gap in the literature in terms of clarifying their effects and at which concentrations and in which cell groups their effects are more effective. This study will fill the gaps in the literature and contribute to these re-evaluations.

In a study conducted in 2018, the genotoxicity of NaA was investigated under *in vivo* conditions, and a significant genotoxic and cytotoxic effect was detected. It was stated that the release of reactive oxygen species (ROS) by acetate may be a mechanism underlying the cyto-genotoxic effect of NaA (Abd-Elhakim et al., 2018). Therefore, the genotoxic and cytotoxic effects observed in this study may be due to the release of ROS. The resulting ROS and free radicals can react with multiple vital components of the cell, such as proteins, lipids, and DNA, and disrupt the structure and function of these components. Free radicals are unstable, low molecular weight, and very active molecules. Since free radicals are highly reactive compounds, they carry unpaired electrons in their outermost orbitals and quickly react with other organic and inorganic molecules. Free radicals are unstable and react with many organic molecules in the cell, like DNA, to produce various damages that may cause abnormalities (Mercan, 2004; Hou et al., 2024).

Conclusion

Food preservatives are the most commonly used group of food additives. Extending shelf life and reaching the consumer without spoilage are among the most essential concerns in food production. Therefore, food additives have become indispensable in the prepared and packaged food industry. This situation has made exposure to food additives inevitable. However, the potential health risks of these chemicals have always been a matter of debate. The point to be considered is to minimise the hazards as much as possible by ensuring that these substances are used as specified and safely. To protect human health, it is essential to consider the food production chain as a system to continuously control food safety and evaluate scientific evidence and all kinds of risk assessments in all measures taken to protect consumer interests.

Results of this study showed that NaA and NaS exhibited a cyto-genotoxic effect at high concentrations. Different studies indicate that this effect may be due to free radical formation. Free radicals, which are low in most foods, increase depending on the processing technique of foods. In other words, the free radical content of processed food is higher than the content of the raw material. The formed ROS and free radicals can react with multiple vital components of the cell, such as protein, lipid, and DNA, and disrupt the structure and function of these components. Considering other studies with NaA and NaS, caution should be exercised when using NaA and NaS because of the cyto-genotoxic effect in different tissues with different tests supporting each other. However, further studies with various test methods and cell groups are believed to provide direction on the level of hazard associated with using these food additives.

Compliance with Ethical Standards

Conflict of interest: The author(s) declares that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: This study was conducted with permission numbered 2023/121 obtained from the Amasya University Ethics Committee.

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