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The Effects of Sodium Nitrate and Propyl Gallate Preservatives on Inducing Chromosomal Abnormalities in Human Lymphocytes

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ABSTRACT

Background and Objective: This study aims to evaluate the potential of two preservatives, sodium nitrate and propyl gallate, to induce chromosomal abnormalities in human lymphocytes.

Materials and Methods: The effects of sodium nitrate at concentrations of 500, 1000, and 2000 ppm, and propyl gallate at concentrations of 1, 5, and 10 ppm, were assessed on human lymphocytes in vitro over exposure periods of 24, 48, and 72 hours. The micronucleus assay was employed to detect chromosomal damage. Additionally, chromosomal breakage tests were performed to verify chromosomal abnormalities by analyzing metaphase spreads for visible chromosomal breaks.

Results: After 59 hours of exposure to sodium nitrate, chromosomal damage increased dose-dependently. Although cells showed a gradual decrease in mitotic divisions, this reduction was insufficient to prevent chromosomal damage, resulting in dose-dependent increases in chromosomal abnormalities. At 91 hours of treatment, cells further reduced mitotic activity, presumably to facilitate repair or elimination of damaged cells; however, the chromosomal damage was too severe for cells to prevent injury, leading to continued increases in chromosomal abnormalities with higher doses. For propyl gallate, an increase in chromosomal damage was observed after 24 hours of treatment with increasing doses. Interestingly, after 40 hours, chromosomal damage and micronuclei frequency decreased with increasing dose. Chromosomal breaks were frequently observed throughout the treatments.

Conclusion: The findings suggest that sodium nitrate and propyl gallate, at the tested concentrations and exposure durations, exhibit genotoxic effects on human lymphocytes.

Key words: Chromosomal abnormalities, preservatives, sodium nitrate, propyl gallate

INTRODUCTION

Cancer is a disease influenced by both genetic and environmental factors. Around 5 to 10 percent of cancers are entirely hereditary and attributed to genetic causes, while 90 to 95 percent of cases are linked to environmental factors. Environmental factors include not only elements outside of genetics but also extend beyond air, water, and soil to encompass conditions and substances found in the home and workplace. These include diet, smoking, alcohol and drug use, exposure to chemicals, sunlight, ionizing radiation, electromagnetic fields, infectious agents, lifestyle choices, as well as economic and behavioral factors—

essentially covering all aspects of our surroundings. Common environmental contributors to cancer-related mortality include tobacco use (approximately 25-30%), diet and obesity (30-35%), infections (15-20%), ionizing and non-ionizing radiation (over 18%), stress, physical inactivity, environmental pollution, and others (Jemal et al., 2011).

There are various theories regarding the origin of cancer, among which the role of chromosomal abnormalities in cancer development is undeniable (Malvandi et al., 2007). Aneuploidy is one of the most common mechanisms involved in the occurrence of cancer. Multiple mechanisms contribute to the generation of aneuploid cells during mitosis, such as chromosome missegregation and chromosome loss. When sister chromatids fail to separate properly and move to opposite poles during cell division, this ultimately results in one nucleus with a missing chromosome and another nucleus with an extra chromosome. The chromosomal variability caused by aneuploidy is recognized as a mechanism that significantly increases the likelihood of cancer occurrence—a rare and stochastic event. Theodor Boveri was the first to observe that abnormal nuclear morphology and mitoses are present in cancer (Boveri, 2008).

Since cancers exhibit aneuploid and clonal karyotypes, and because these facts cannot be adequately explained by the traditional mutation theory, Nicholson and Duesberg proposed the karyotypic theory of cancer in a study. According to this theory, carcinogens initiate cancer by inducing random aneuploidy, which then catalyzes changes in the cell's karyotype. Ultimately, the tumor progresses spontaneously to malignancy. The alteration in the karyotype occurs because aneuploidy disrupts the balanced groups of proteins involved in chromosome segregation, synthesis, and repair (Nicholson and Duesberg, 2009).

Since approximately 30-35% of cancers are related to diet, identifying and controlling factors that increase chromosomal damage is essential. One such factor that induces these disorders is food preservatives, which are widely used in food products. Therefore, investigating and studying the effects of food preservatives on the human genome is vital. Preservatives are substances used to maintain food quality, reduce spoilage (loss of quality or nutritional value), and prevent decomposition caused by microorganisms. These substances can be added at various stages including production, packaging, transportation, or storage of food items.

Propyl Gallate (PG) is a compound commonly used in combination with BHT and BHA and is found in many meat products, including chicken soup, and more recently, in chewing gum. Propyl gallate is the *n*-propyl ester of gallic acid and functions as a free radical scavenger with antioxidant properties. Since 1948, this antioxidant has been added to foods containing oils and fats to prevent oxidation. It protects food materials against oxidation by hydrogen peroxide and free radicals. Free radicals are molecules that lack a complete electron shell, which makes them highly reactive chemically compared to other molecules (Gálico et al., 2015). The biological activity of propyl gallate is based on its ability to inhibit free radicals. Its effects include antimicrobial activity, enzyme inhibition, suppression of biosynthetic processes, inhibition of nitrosamine formation, anesthetic effects, inhibition of neuromuscular responses to chemicals, and protection against ultraviolet (UV) radiation.

Sodium Nitrate is a common preservative, coloring agent, and flavor enhancer used in bacon and hot dogs. Nitrate (NO_3^-) is a white crystalline powder naturally present in our environment. It has been shown that nitrates are also found in fruits and vegetables (Sanchez-Echaniz et al., 2001). The nitrates in vegetables can be converted into nitrites by bacteria in the mouth—specifically nitrate reductase bacteria located under the tongue (Webb and Lidder, 2013). Sodium nitrate and sodium nitrite are closely related, and both are used as preservatives in processed meats. Besides their antimicrobial properties at low concentrations, nitrite compounds are also used to develop color, flavor, and aroma. In fact, nitrite and nitrate compounds are added to prevent the growth of microorganisms, especially *Clostridium botulinum*, and to create the pink color and desirable taste in processed meats. Nitrite alone exhibits antimicrobial activity, while nitrate shows this effect

only after being reduced to nitrite. The exact mechanism by which nitrite affects bacteria is not fully understood, but it is believed that nitrite interacts with sulfhydryl groups in microorganisms, causing modifications that disrupt metabolism and ultimately inhibit microbial growth and proliferation.

According to statistics, the highest percentages of chromosomal abnormalities were observed respectively with propyl gallate (82.72%), sodium nitrate (44.81%), sorbic acid (43.67%), butylated hydroxytoluene (15.60%), and butylated hydroxyanisole (12.93%) (Himadri Pandey et al., 2014). According to the study by Mamur Sevcan (2012), the food preservative sodium sorbate can induce chromosomal abnormalities in human lymphocytes. Furthermore, research by Himadri and colleagues (2014) demonstrated that five compounds—sodium nitrate, propyl gallate, sorbic acid, butylated hydroxytoluene, and butylated hydroxyanisole—commonly used in food packaging, exhibited genotoxic effects on the chromosomes of the plant *Allium cepa*, which may also be harmful to other organisms, particularly humans. Since the effects of the two preservatives sodium nitrate and propyl gallate on the induction of chromosomal abnormalities in humans have not yet been studied, the present research aimed to examine the effects of sodium nitrate at concentrations of 500, 1000, and 2000 ppm and propyl gallate at concentrations of 1, 5, and 10 ppm on human lymphocytes in vitro over exposure periods of 24, 48, and 72 hours. To achieve this, the micronucleus assay, which offers greater speed and efficiency compared to other methods for detecting chromosomal damage, was used. Additionally, to confirm chromosomal damage, chromosomal breakage tests were performed, metaphase spreads were carefully examined, and chromosomal breaks were observed.

Materials and Methods

In this study, blood samples were collected from two individuals (one male and one female) who met the inclusion criteria (the number and gender of participants were selected based on similar studies). The inclusion criteria were: no history of genetic diseases or chromosomal abnormalities in the family pedigree, an age range of 20 to 30 years, no history of illness or use of specific medications, non-smoking status, and no exposure to radiological radiation for at least the past three months. After obtaining informed consent and completing consent forms, blood collection was performed by a trained phlebotomist using 5 mL syringes. The blood samples were transferred to heparinized tubes and stored in a refrigerator until lymphocyte cell culture could be conducted. It should be noted that 5 mL of blood was collected from each participant at every stage of the study.

First, 100 microliters of colcemid were added to each culture, and the tubes were incubated for 18 minutes. Then, the tubes were centrifuged at 1200 RPM for 10 minutes. After centrifugation, the supernatant was discarded, and 10 milliliters of 37°C potassium chloride (KCl) solution were added. To homogenize the contents, pipetting was performed thoroughly. The tubes were then incubated at 37°C for 30 minutes. Next, the tubes were centrifuged again at 1200 RPM for 10 minutes. The supernatant was discarded, and the remaining pellet was gently resuspended by tapping with fingers to form a suspension. Cold fixative solution consisting of methanol and acetic acid in a 3:1 ratio was gently added to reach a final volume of 10 milliliters. The suspension was homogenized again by pipetting and centrifuged at 1200 RPM for 10 minutes. In the final steps, the supernatant was discarded, and the pellet was resuspended in 6 milliliters of fixative. The tubes were centrifuged again at 1200 RPM for 10 minutes. Finally, after removing the supernatant, the pellet was resuspended in 2 milliliters of fixative.

In the micronucleus assay, to assess the genotoxicity of the test agents (in this study, the selected preservatives), the percentage of micronuclei is evaluated. To assess cytotoxicity, the cell division index (BI index) is measured. Evaluating the division index alongside the micronucleus test greatly aids in the interpretation of results. For each treatment, 3 to 4 slides were prepared. Approximately 500 cells were

counted per slide.

The obtained results were analyzed using SPSS software with the Mann-Whitney test. A significance level of 0.05 was considered. Finally, graphs were plotted using Excel software. Chromosomal breakage analysis was performed with the aid of karyotyping to confirm chromosomal damage observed in the micronucleus assay. For each treatment, 20 metaphase spreads were photographed and analyzed for chromosomal abnormalities.

Findings

Microscopic Observations from the Micronucleus Assay

In this study, the micronucleus assay was used to evaluate chromosomal damage induced by the food preservatives sodium nitrate and propyl gallate in human binucleated lymphocytes. Following various treatments and Giemsa staining, different cell types—including mononucleated cells, binucleated cells without micronuclei, and binucleated cells with micronuclei—were counted at 100x magnification on each slide.

As shown in Figure 1, cells in the control group, treated only with cytochalasin B, remained binucleated. In contrast, cells treated with preservatives exhibited not only two main nuclei but also micronuclei (Figure 2).

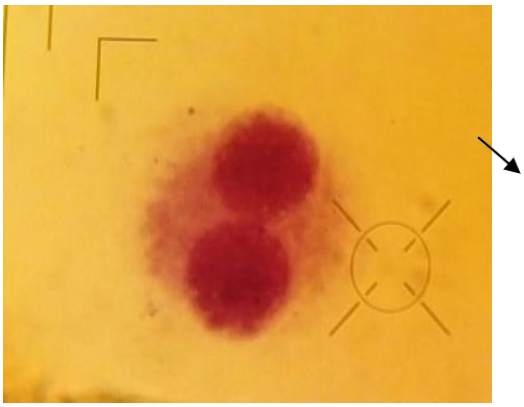

Figure 1: Stained binucleated lymphocytes. The arrowhead indicates the cytoplasm, which appears as a faint halo.

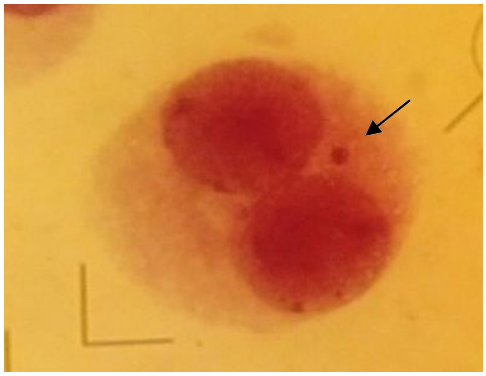

Figure 2: Micronucleus in a binucleated lymphocyte. The arrowhead points to the micronucleus.

Effect of Gender (Male and Female) on Micronucleus Test Results

In 2012, Mamur Sevcan and colleagues used lymphocyte samples from two healthy individuals (one male and one female) to assess the genotoxicity of sodium sorbate on human lymphocytes. Following this approach, this study also selected one healthy male and one healthy female as samples.

The comparison of micronucleus test results for treatments with sodium nitrate and propyl gallate, separated by gender, is presented in Figures 3 and 4.

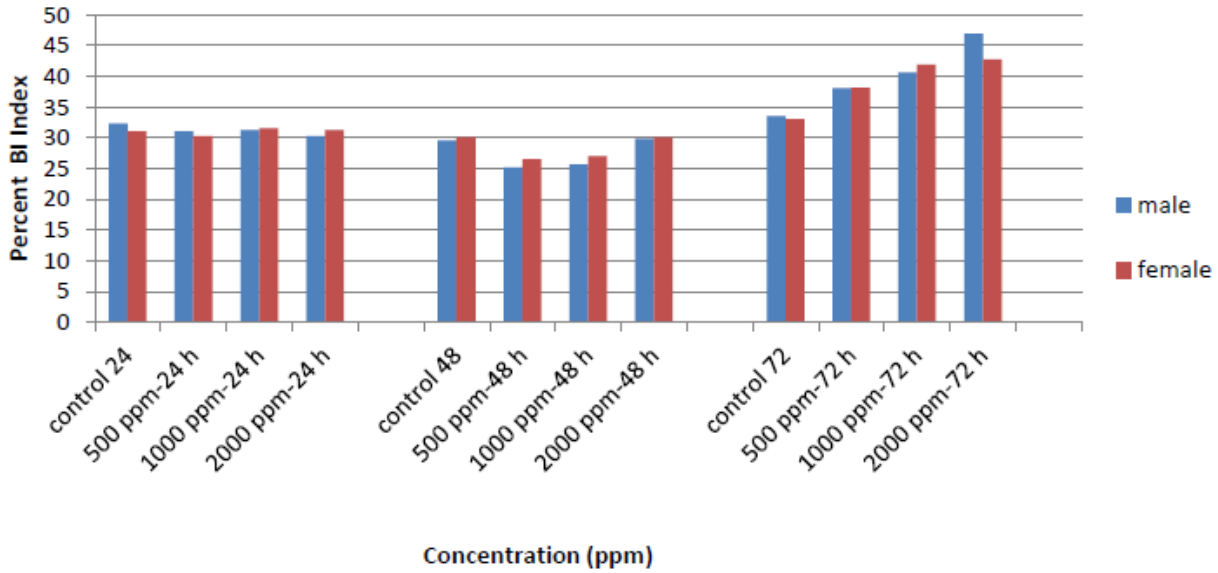

Figure 3: Comparison of the division index between male and female following treatment with sodium nitrate.

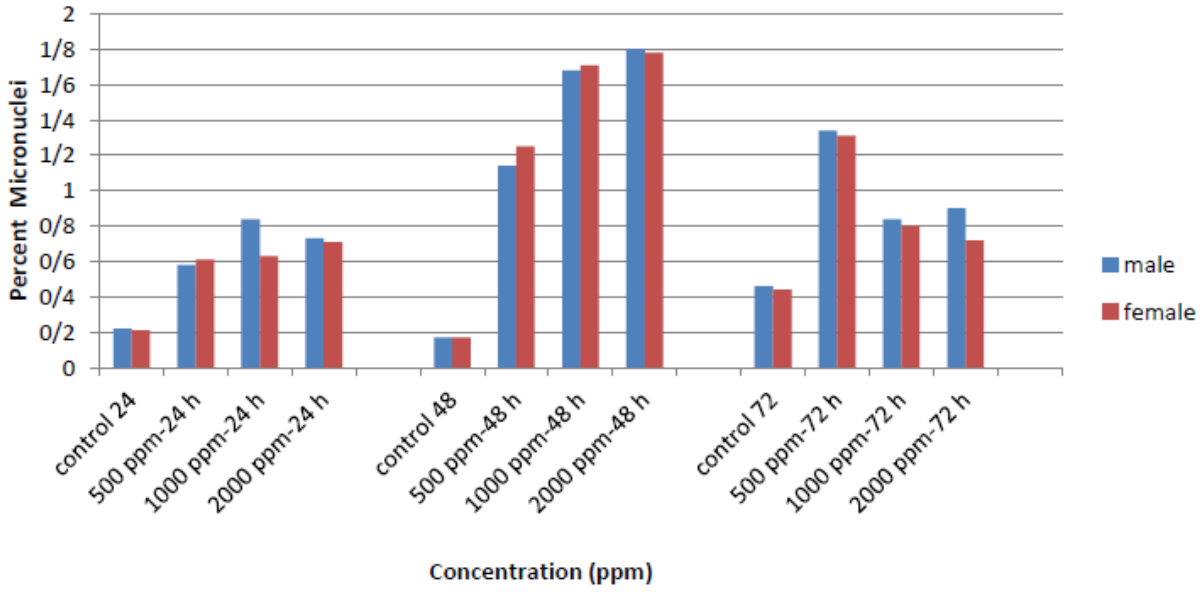

Figure 4: Comparison of micronucleus frequency between male and female following treatment with sodium nitrate.

As shown in Figures 3 and 4, there is no significant difference in the division index and micronucleus frequency between males and females treated with sodium nitrate.

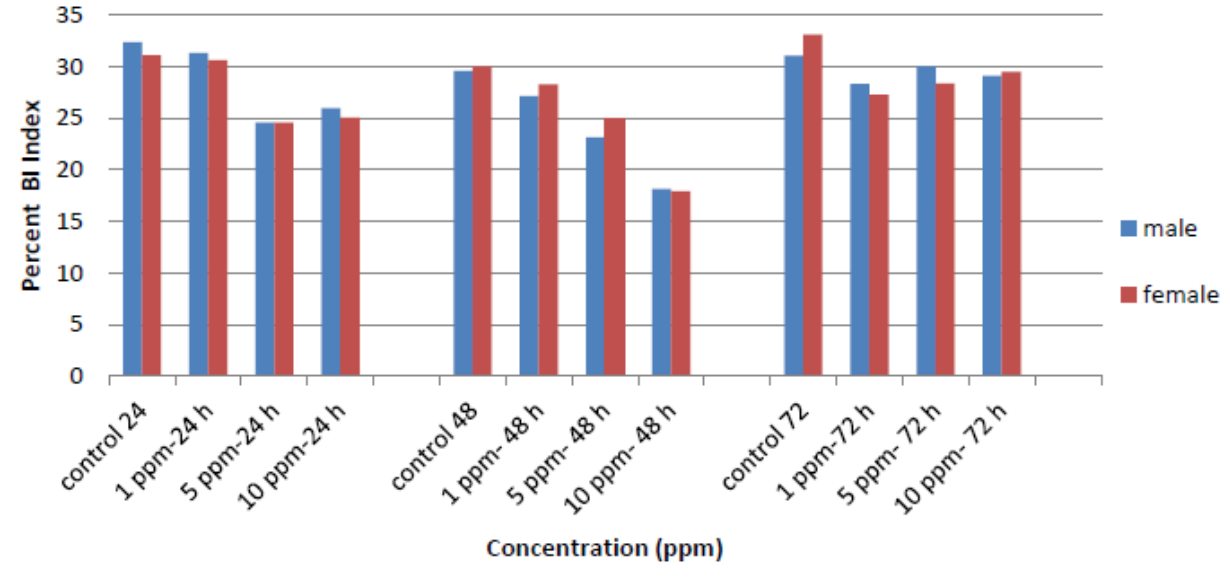

Figure 5: Comparison of the division index between male and female following treatment with propyl gallate.

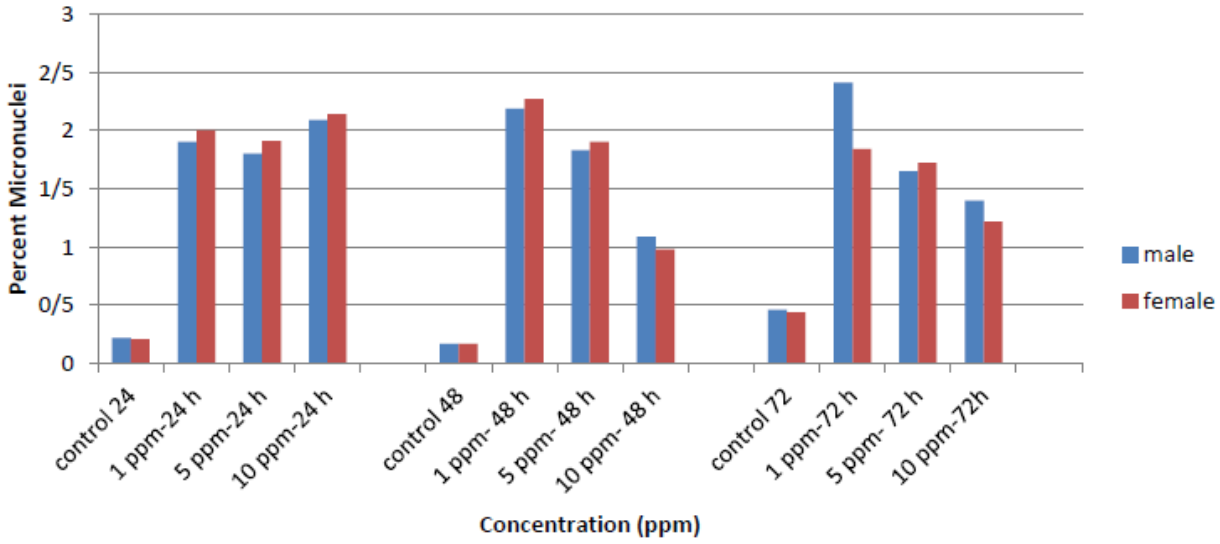

Figure 6: Comparison of micronucleus percentage between male and female following treatment with propyl gallate.

According to Figures 5 and 6, the effect of propyl gallate on the division index and micronucleus percentage is nearly the same between males and females. Given that no significant differences in chromosomal damage caused by sodium nitrate and propyl gallate were observed between the two genders, reporting the micronucleus assay results for only one gender (male) appears sufficient.

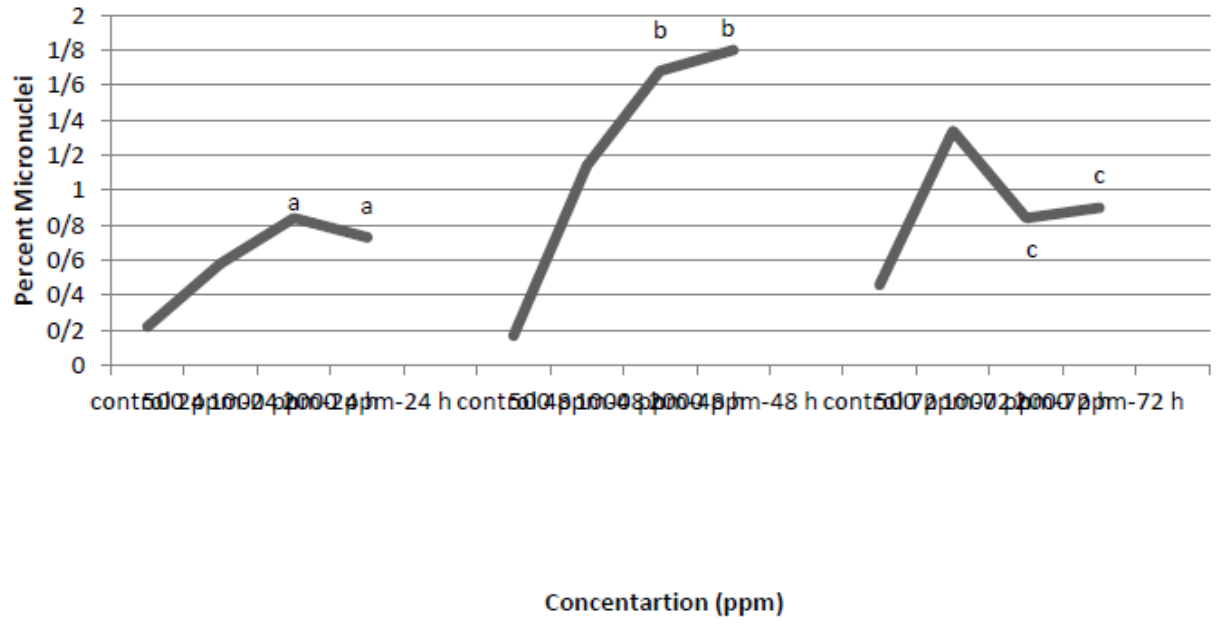

Figure 7: Changes in micronucleus percentage in binucleated cells (male) treated with sodium nitrate. Identical symbols indicate no significant difference.

As shown in Figure 7, at 24 and 48 hours of treatment with sodium nitrate, chromosomal damage increases with increasing dose, although there is no significant difference between the 1000 ppm and 2000 ppm doses. Additionally, after 72 hours of treatment, chromosomal damage decreases with increasing dose, but the reduction between 1000 ppm and 2000 ppm is not statistically significant.

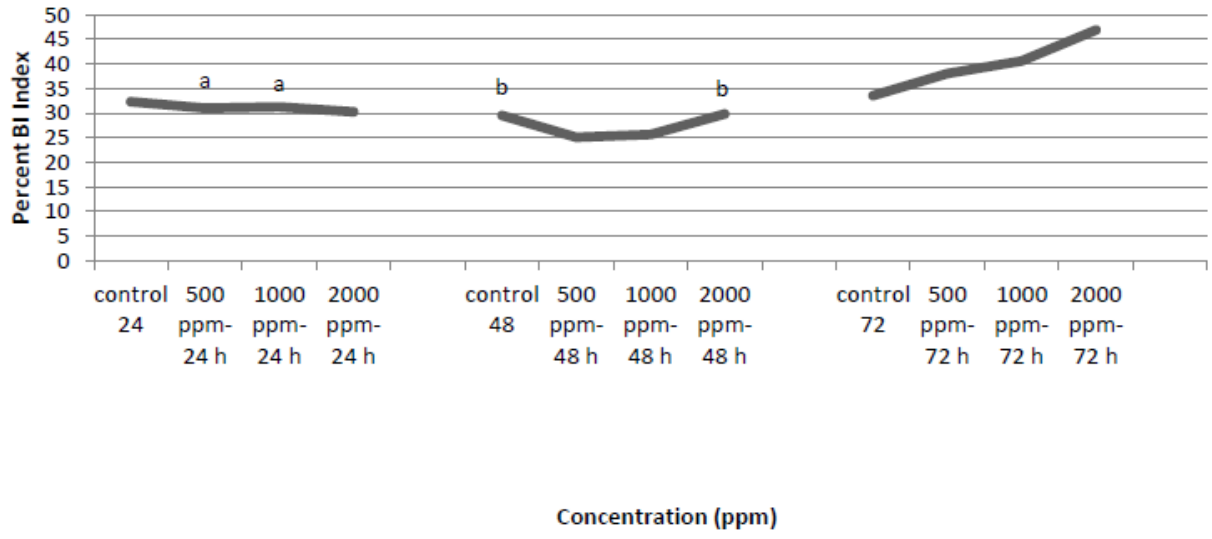

Figure 8: Changes in the cell division index (BI Index) in binucleated cells (male) treated with sodium nitrate. Identical symbols indicate no significant difference.

As shown in Figure 8, the division index after 24 hours of sodium nitrate treatment shows a slight decrease compared to the control, with no significant difference between 500 ppm and 1000 ppm doses. After 48 hours of treatment, the division index initially decreases, then increases with higher doses to a level that is not significantly different from the control. Following 72 hours of treatment, the division index increases

significantly compared to the 72-hour control.

Table 1: Mean percentage of micronuclei and division index for sodium nitrate treatment on the male sample.

Tests (SN) male	Control 24 h	500 ppm- 24 h	1000 ppm- 24 h	2000 ppm- 24 h	Control 48 h	500 ppm- 48 h	1000 ppm- 48 h	2000 ppm- 48 h	Control 72 h	500 ppm- 72 h	1000 ppm- 72 h	2000 ppm- 72 h
MI Mean	0.22	0.58	0.84	0.73	0.17	1.14	1.68	1.8	0.46	1.34	0.84	0.9
BI Mean	32.3	31	31.25	30.24	29.53	25.11	25.64	29.83	33.56	38.06	40.62	46.9

Table 2: Mean percentage of micronuclei and division index for sodium nitrate treatment on the female sample.

Tests (SN) female	Control 24 h	500 ppm- 24 h	1000 ppm- 24 h	2000 ppm- 24 h	Control 48 h	500 ppm- 48 h	1000 ppm- 48 h	2000 ppm- 48 h	Control 72 h	500 ppm- 72 h	1000 ppm- 72 h	2000 ppm- 72 h
MI Mean	0.21	0.61	0.63	0.71	0.17	1.25	1.71	1.78	0.44	1.31	0.8	0.72
BI Mean	31.05	30.21	31.5	31.24	30	26.54	27	30	33.04	38.11	41.87	42.69

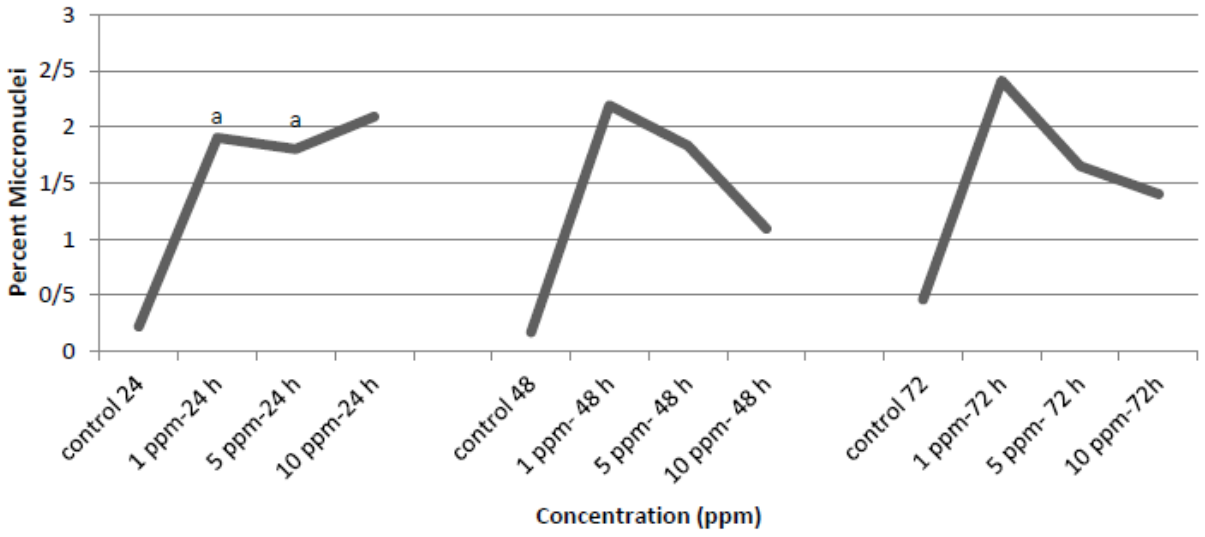

Figure 9: Changes in micronucleus percentage in binucleated cells (male) treated with propyl gallate.

As shown in Figure 9, after 24 hours of treatment with propyl gallate, chromosomal damage increases with increasing dose. At 24 hours and 5 ppm dose, a decrease in damage is observed; however, this decrease is not statistically significant compared to the 1 ppm dose. In the 48- and 72-hour treatments, chromosomal

damage decreases with increasing dose.

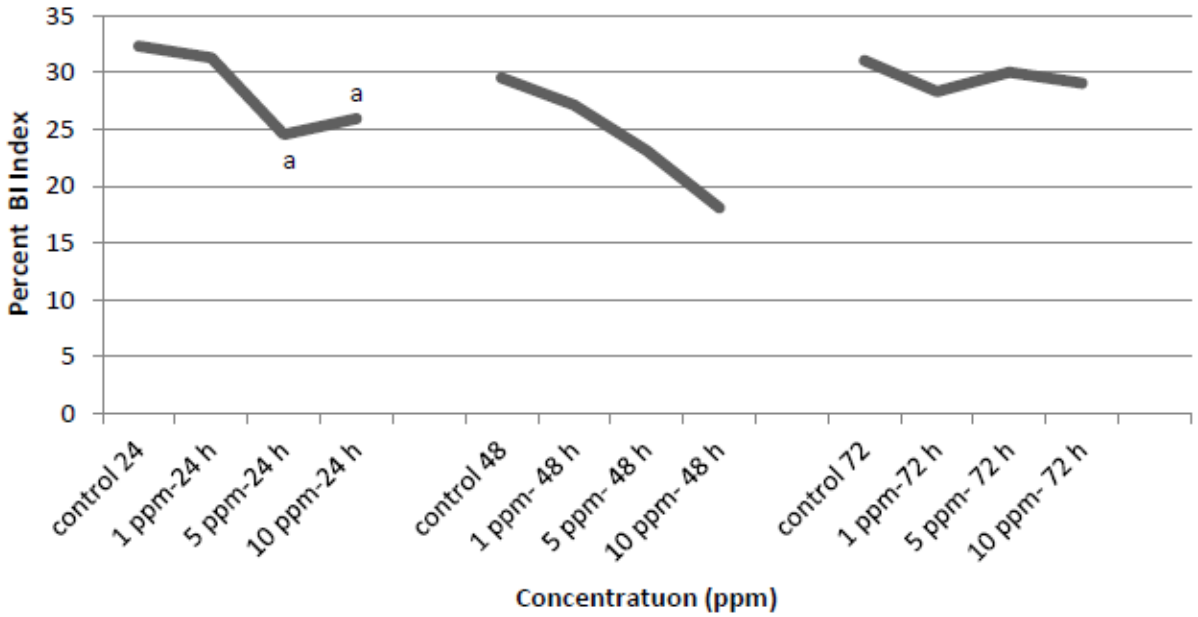

Figure 10: Changes in the cell division index (BI Index) in binucleated cells (male) treated with propyl gallate.

According to the results in Figure 10, a decrease in the division index is observed at 24 and 48 hours of treatment with propyl gallate, which shows a direct relationship with increasing dose. It is worth noting that the increase in BI at 24 hours with a 10 ppm dose is not significantly different from the same treatment duration at 5 ppm. After 72 hours of treatment, an increasing trend in the division index is observed with increasing dose.

Table 3: Mean percentage of micronuclei and division index for propyl gallate treatment on the male sample.

Tests (PG) male	Contr ol 24 h	500 ppm- 24 h	1000 ppm- 24 h	2000 ppm- 24 h	Contr ol 48 h	500 ppm- 48 h	1000 ppm- 48 h	2000 ppm- 48 h	Contr ol 72 h	500 ppm - 72 h	1000 ppm - 72 h	2000 ppm - 72 h
MI Mean	0.22	1.9	1.8	2.09	0.17	2.19	1.83	1.09	0.46	1.77	1.65	1.4
BI Mean	32.3	31.27	24.54	25.93	29.53	27.13	23.11	18.09	31.02	28.31	30	29.05

Table 4: Mean percentage of micronuclei and division index for propyl gallate treatment on the female sample.

Tests (PG) Female	Control 24 h	500 ppm- 24 h	1000 ppm- 24 h	2000 ppm- 24 h	Control 48 h	500 ppm- 48 h	1000 ppm- 48 h	2000 ppm- 48 h	Control 72 h	500 ppm- 72 h	1000 ppm- 72 h	2000 ppm- 72 h
MI Mean	0.21	2	1.91	2.14	0.17	.27	1.9	0.98	0.44	1.84	1.72	1.22
BI Mean	31.05	30.57	24.55	25.02	30	28.24	24.98	17.89	33.04	27.22	28.32	29.44

Results of Chromosomal Breakage Analysis

To confirm the micronucleus assay and verify the induction of chromosomal damage, chromosomal breakage analysis was performed, and metaphase spreads were examined for chromosomal abnormalities. Figure 11 shows a metaphase spread from an untreated (control) sample. In Figure 12, the sites of chromosomal damage are indicated by arrowheads.

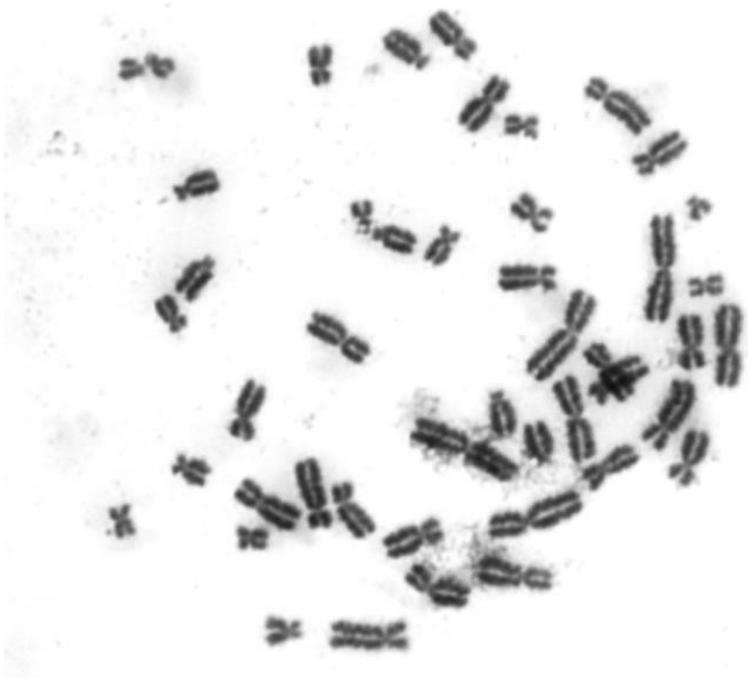

Figure 11: Metaphase spread of untreated (control) cells.

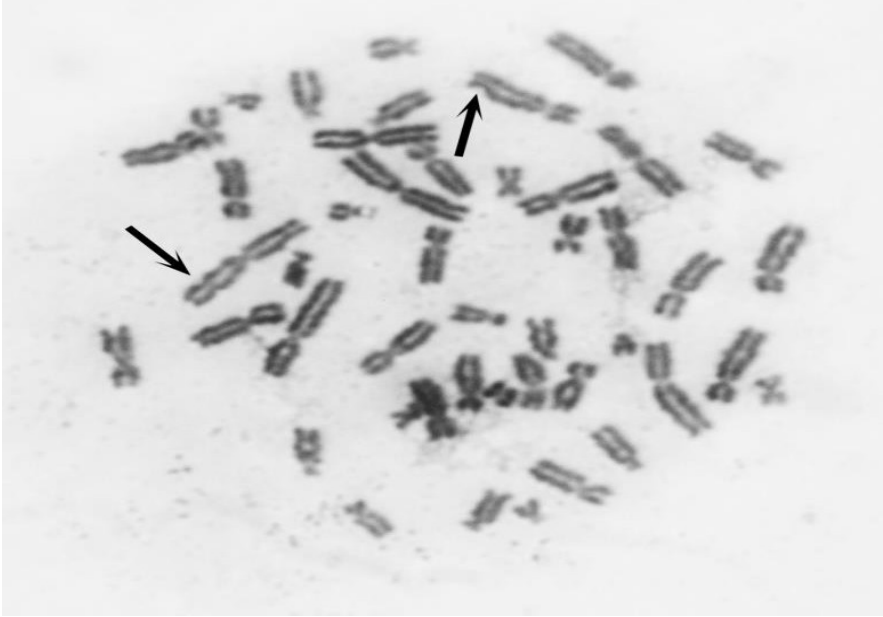

Figure 12: Metaphase spread of treated cells. Arrows indicate the sites of chromosomal damage.

Discussion

The results showed that after 24 hours of treatment with sodium nitrate, chromosomal damage increased with rising doses. Meanwhile, the cells gradually reduced their rate of division; however, this decrease in cell division was insufficient to prevent chromosomal damage, leading to a dose-dependent increase in chromosomal abnormalities. After 48 hours of treatment with sodium nitrate, cells further reduced mitotic division in an attempt to repair or eliminate damaged cells. Nonetheless, the chromosomal damage was so severe that the cells could not prevent further harm, and consequently, an increase in chromosomal damage was observed with increasing doses.

After 72 hours of treatment, the frequency of micronuclei decreases due to the removal or repair of damaged cells, indicating the instability of the chromosomal damage inflicted. On the other hand, cell division increases significantly with higher doses, which may be related to the mitogenic properties of sodium nitrate. This hypothesis could be further investigated using MTT assays in future studies. Alternatively, the increase in cell division after 72 hours of treatment might result from the disruption of synchronized cell division in the culture environment. Prolonged cell culture can disturb the regularity of cell cycles. Cells typically divide in a regular and synchronized manner during the first 72 hours in culture, but after this period, synchronization is lost. This loss of coordination may explain the observed increase in cell division after 72 hours, though further research is needed to confirm this.

The results showed that after 24 hours of treatment with propyl gallate, chromosomal damage increased with rising doses. Since chromosomal damage can lead to cell cycle arrest at checkpoint points or induce apoptosis via the activity of TP53 and P21 proteins, the cells initiated a defensive response during this 24-hour treatment by reducing cell division (decreased BI index) to try to control the damage. However, this reduction in cell division was insufficient, and the cells were unable to effectively combat the induced damage, resulting in a dose-dependent increase in chromosomal abnormalities.

After 48 hours of treatment with propyl gallate, chromosomal damage decreased and the frequency of

micronuclei was reduced with increasing dose. This is because the cells strongly suppressed division to prevent further damage and were able to repair or eliminate damaged cells. Following 72 hours of treatment, the cells adapted to the conditions, and the binucleated (healthy) cells resumed division, returning close to normal levels. Given the observed decrease in micronucleus frequency, it can be concluded that the genetic damage was not persistent, and the cells effectively repaired the damage or removed the damaged cells.

After performing the micronucleus assay, chromosomal breakage analysis was conducted to confirm the chromosomal damage induced by treatment with sodium nitrate and propyl gallate. Since micronuclei can arise either from chromosomal breakage that produces acentric fragments released into the cytoplasm (structural abnormalities) or from chromosomes that lag during anaphase causing numerical abnormalities (aneuploidy), metaphase spreads were examined for structural and numerical chromosomal abnormalities to determine the origin of the micronuclei. The results showed abundant chromosomal breaks, although no aneuploidy was observed. Due to the extensive scope of the study and time constraints, definitive conclusions could not be drawn, and further detailed investigation using techniques such as FISH (Fluorescence In Situ Hybridization) is needed to accurately identify the origin of the observed micronuclei.

According to the results of Hasegawa and colleagues, sorbic acid and its potassium salts induce chromosomal abnormalities and sister chromatid exchanges in Chinese hamster cells (Hasegawa et al., 1984). Additionally, Rencuzogullari and coworkers reported that sodium metabisulfite causes a reduction in the division index in human lymphocytes and Allium plants (Rencuzogullari et al., 2001).

In another study conducted in 2008, it was found that sodium phosphate caused a significant decrease in the cell division index and an increase in chromosomal abnormalities in the Marigold plant (Romeo-Cristian et al., 2008). Subsequently, in 2011, the effects of sodium benzoate and potassium benzoate on human lymphocytes were investigated, and the results demonstrated the mutagenic, clastogenic, and cytotoxic properties of these two compounds on human lymphocytes (Zengin et al., 2011).

In a 2012 study conducted by Mamur Sevcan and colleagues investigating the genotoxicity of sodium sorbate on human lymphocytes, the results demonstrated that sodium sorbate can induce genotoxic effects in human lymphocytes. In this study, human lymphocytes were exposed to varying concentrations of sodium sorbate (100, 200, 400, and 800 ppm) for 24 and 48 hours. The findings indicated that sodium sorbate significantly induced chromosomal abnormalities, including gaps, chromosomal breaks, chromatid breaks, sister chromatid exchanges, dicentric chromosomes, and acentric fragments. Additionally, polyploidy, a numerical chromosomal abnormality, was also observed. Based on these results, sodium sorbate at the tested concentrations has the capacity to induce both structural and numerical chromosomal aberrations in human lymphocytes (Mamur Sevcan et al., 2012).

Subsequently, in 2014, a study was conducted on five commonly used preservatives in the United States, including propyl gallate, sorbic acid, sodium nitrate, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA). In this study, the roots of the plant *Cepa Allium* (from the onion family) were treated with varying concentrations (1000, 1500, 2000, and 2500 ppm) of these compounds.

Cepa Allium is one of the best standard plant models that demonstrates cytogenetic changes and is closely related to mammalian systems. The results of this study confirmed that all five compounds were capable of inducing chromosomal abnormalities, mitotic irregularities, changes in the frequency of cells at different cell cycle stages, and the formation of necrotic cells in *Cepa Allium*. The extent of abnormalities was dependent on the concentration and duration of treatment. Statistical analysis in this research showed that the smallest decrease in the mitotic index was observed with sodium nitrate treatment, whereas chromosomal abnormalities were more pronounced in cells treated with this compound compared to the others. It is likely that the reduction in the mitotic index is due to inhibition of DNA synthesis or cell cycle arrest in the G2

phase, which prevents completion of mitosis in the cells.

Conclusion

Based on the results of this study and the observed chromosomal damage detected through the micronucleus assay, as well as karyotyping analysis of cells treated with sodium nitrate and propyl gallate, it can be concluded that these two compounds exhibit genotoxic effects on human lymphocytes at the doses and exposure times used in this research.

Given these findings, it is recommended to extend the investigation to other cell types besides blood cells, which have different growth and division rates. Additionally, evaluating induced damage at time points shorter than 24 hours and longer than 72 hours, testing doses both lower and higher than those used here, and conducting in vivo studies using laboratory animals such as mice are suggested for a more comprehensive assessment.

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