Studying the genotoxicity caused by sodium benzoate in liver cancer cell line (HEPG2) by comet assay

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Abstract---Objective: Genetic toxicity examines the health of those exposed to mutagenic agents and the mutagenic impacts of radiation and chemicals. The process of causing genetic alterations and DNA damage is known as mutagenesis. Therefore, it is vital to carry out research in this area at many levels and come up with suitable ways to stop or lessen the development of genetic toxicity. The sodium version of benzoic acid, sodium benzoate, is a common ingredient in both food and cosmetics. This study aims to explore the genotoxicity produced by sodium benzoate in the liver cancer cell line (HepG2) using the Comet assay because of the substance’s widespread use.

Method: The comet assay or single cell gel electrophoresis (SCGE) is a quick and accurate method for identifying and assessing DNA damage in single cells. One-way ANOVA and Tukey’s multiple comparison posthoc test were used to examine the results, which were shown as the SD Mean of the report and the data connected to the Comet indices. The difference’s significance threshold was set at p<0.0001.

Findings: DNA damage was generated in the liver cancer cell line in the current investigation by sodium benzoate’s genotoxicity at concentrations of 50, 100, and 150 micromolar (HepG2). Additionally, it decreased intracellular glutathione reduction and raised reactive oxygen species (ROS). Conclusion: Compared to the control group, sodium benzoate significantly damaged DNA.

Keywords---genotoxicity, sodium benzoate, HepG2 cancer cell, Comet assay.


**Introduction**

A common ingredient in cosmetic goods is sodium benzoate, which is the sodium salt of benzoic acid. This chemical is employed as a preservative in the field of food chemistry to stop the development of germs (Kundu et al., 2016). Food spoiling is avoided because of sodium benzoate's ability to stop the growth of bacteria, mold, and other organisms. This works particularly well with acidic meals. In order to avoid this, it is typically utilized in foods like soda, bottled lemon juice, pickles, jellies, salad dressing, soy sauce, and other spices (Nettis et al., 2004). Many cosmetics, including toothpaste, mouthwash, baby wipes, and hair products, employ sodium benzoate as a preservative (Basch et al., 2016).

According to animal research, the amount of sodium benzoate taken can directly trigger the body's inflammatory pathways. Among them is the development of cancerous inflammation (Raposa et al., 2016). A tiny percentage of persons who consume meals or use personal care items containing sodium benzoate may have adverse responses like hives and edema after doing so (Moyano et al., 1996). On the other hand, a guy with severe depression got 500 mg of sodium benzoate every day for six-week research. These patients' symptoms improved by 64%, and an improvement in depression-related brain structure was seen on their MRI scans (Lai, 2013). Within six weeks of receiving 500 mg of sodium benzoate daily, panic symptoms in women with panic disorder that included anxiety, stomach discomfort, tightness in the chest, and palpitations decreased by 61 percent (Hou et al., 2013). Sodium benzoate may have certain advantages, but it can also have negative effects, including nausea, vomiting, and discomfort in the abdomen (Misel et al., 2013).

Single-cell gel electrophoresis (SCGE), often known as the comet assay, is a quick and accurate method for identifying and assessing DNA damage in individual cells. In order to assess genetic damage and the efficiency of medications used in chemotherapy protocols, this approach is one of many employed in cancer research (Eskander HG, et. al., 2021; Alhomayani FK, et. al., 2020; Alsayed MA, et. al., 2019). In 1984, Swedish researchers Ostling & Johansson created this method. In 1988, Singh and his associates altered the comet test and gave it the moniker Alkaline Comet Assay (McKelvey-Martin et al.). Single and double chain breakage, oxidative stress damage, and the formation of crosslinks between DNA and DNA proteins are all investigated using the Comet assay. The monitoring of DNA repair in live cells is also done using this technique.

Comet assay has been used with several cell types, including CHO, V79, and HepG2. In this investigation, a HepG2 cell line was utilized (Fairbairn et al., 1995). A 15-year-old boy's hepatoblastoma yielded hepG2 cells, which were later employed in a number of mutagenicity investigations. HepG2 cells have an epithelial appearance and 55 pairs of chromosomes. Due to the expression of liver phase II and I enzymes, HepG2 is an excellent model for many sorts of toxicological research (Westerink et al., 2007).

In a research published in 2016, Bjoraker et al. looked at how sodium benzoate and dextromethorphan therapy affected individuals with a mild type of non-tectotic hyperglycemia. They discovered that in infants with a mild illness caused
by mutations with residual activity, early therapy with dextromethorphan and sodium benzoate to regulate plasma glycine levels is useful in improving prognosis. Beezhold et al. (2014) investigated the association between student symptoms of ADHD and consumption of a beverage high in sodium benzoate. According to the research, drinking beverages high in sodium benzoate was significantly associated with ADHD symptoms ($p = 0.001$). In animal research, Raposa et al. (2016) shown that sodium benzoate can directly activate the body’s inflammatory pathways depending on the amount taken. Among them is the development of cancerous inflammation (Raposa et al., 2016). In a six-week trial, Lai et al. (2013) discovered that giving a man with severe depression 500 mg of sodium benzoate resulted in a 64% reduction in symptoms and an improvement in brain structure associated with depression (Lai, 2013). In research by Hou et al. (2013) on women with panic disorder who had symptoms including anxiety, stomach ache, tightness in the chest, and palpitations, it was discovered that a daily intake of 500 mg of sodium benzoate reduced panic symptoms by 61 percent in just six weeks (Hou et al., 2013).

In research on sodium benzoate’s high toxicity and deformity in zebrafish larvae, Tsay et al. (2007) demonstrated that gene mutations and alterations in enzyme pathways were to blame for the toxicity of sodium benzoate. Pongsavee et al. (2015) showed that sodium benzoate produced significant genotoxicity by disrupting chromosomes, creating micronuclei, and changing the Ala40Thr superoxide dismutase gene. Noorafshan et al. (2014) reported that sodium benzoate produced neurotoxicity in rats, which is likely connected to neuronal genotoxicity.

Some food colors contain sodium benzoate. These colors may cause hyperactivity when sodium benzoate is added to them (Weiss et al., 1980). The widespread usage of this chemical in food suggests that a more thorough examination of its genetic toxicity in various in vitro and in vivo models is required. As a result, the current work uses the trustworthy Comet assay to examine the genotoxicity produced by sodium benzoate in the HepG2 cancer cell line, a common cell model for examining genetic damage.

**Materials and methods**

**1. Cultivation and maintenance of cells**

One liter of deionized water was received ready, placed into an autoclavable glass container, and sterilized at 120°C and 15 atmospheres of pressure. Deionized water was used to dissolve 2.16 grams of Na2HPO4, 0.2 grams of KH2PO4, 8 grams of NaCl, and 0.2 grams of KCl to create phosphate-buffered saline (PBS). The solution was then autoclaved in an autoclavable glass container for 20 minutes at a pressure of 15 atmospheres and a temperature of 120°C after the pH was adjusted to 7.3-7.6 using NaOH and concentrated HCl. The finished product was kept in the fride (Azarova et al., 2007).

Subsequently, 5 ml Eppendorf flasks of the sterile trypsin solution were split up and kept at -20°C after being purchased frozen (Azarova et al., 2007). Trypan blue
dye was mixed in 100 ml of physiological serum to create a trypan blue solution, which was then stored in screw-capped vials (Azarova et al., 2007).

A sterile 50 ml Falcon was filled with 45 ml of RPMI-1640 media, 500 microliters of antibiotic (penicillin/streptomycin), and 500 ml of sterile fetal calf serum before being mixed to create the complete culture medium. It is preferable to utilize the finished culture medium within a week of production. However, it can be kept in the refrigerator for up to a month. At the same time, all phases of culture medium preparation and cell handling should be carried out in a totally sterile setting and under a hood (Azarova et al., 2007).

2. Solutions required for Comet assay

Lysis solution is also required in addition to PBS solution. The necessary quantities of powdered NaCl, Tris HCl, EDTA, and NaOH were dissolved in deionized water for this purpose. Since Triton X-100 is neutral, it has no effect on pH. After bringing the pH of this solution to between 10 and 10.5, it is necessary to add Triton. The lysis solution was prepared up to 990 ml with deionized water after the aforementioned ingredients had been dissolved due to the lysis solution’s two-week stability without Triton X-100. Half an hour prior to the test, Triton was added to the lysis solution in order to improve quality (Azarova et al., 2007).

Ten regular NaOH and 200 mM EDTA stock solutions were used to make tank buffer. Two hundred grams of NaOH were weighed and diluted in 500 ml of deionized water to create a stock solution of 10 normal NaOH. NaOH effectively draws moisture from the atmosphere. Therefore, after each usage, the container’s lid must be thoroughly closed. 14.8 grams of EDTA powder and 200 mM of deionized water were combined and well agitated to create the stock solution. The solution was then raised to a pH of 10–10.5, and its volume increased to 200 cc before being placed in the refrigerator.

3. Cell culture

In this investigation, the HepG2 cell line was used. The Pasteur Institute of Iran provided this cell in the form of a growth media flask. The entire cell culture process was carried out in an aseptic setting with a laminar hood. The HepG2 cell line has finished consuming the nutrients in the culture media when it completely covers the bottom of the flask. Cells in one flask should be moved to multiple new flasks, and their old culture media should be replaced, a process known as recultivation, in order to prevent cell death at this point. The cells were washed with PBS, and the previous culture medium was discarded before being separated from the flask’s surface with an EDTA-trypsin solution. Fresh growth media was used to counteract the effects of trypsin after the cells had been extracted, and the supernatant was then discarded. The collected cell sediment was mixed with fresh culture media, and after the cell suspension was homogenous, it was split into the number of flasks that were available before being mixed with fresh medium and put in a CO2 incubator (Neeley et al., 2006). The hemocytometer method is a precise and efficient way to count cells, and it may be used to determine how many cells are present per milliliter of solution (Dhar et al., 2002).
The percentage of live cells must be greater than 90% in order to use the Comet assay (Morgan, 2003).

4. Plate preparation and cell and sample proximity

The requisite number of cells must first be produced in order to expose the cells to the proper concentration of sodium benzoate. One milliliter of the cell suspension, which contained 25 104 cells, was added to each well of the 24-well plate after it had been prepared. Calculated sodium benzoate concentrations were introduced to the wells in the appropriate quantities. Each well's total capacity was set at 2 ml. The prepared sodium benzoate has a 300 micromolar concentration. One hundred forty-four grams are the molecular weight of sodium benzoate. The following methods were used to calculate the moles of sodium benzoate.

Table 1: Preparation of different concentrations of sodium benzoate

<table>
<thead>
<tr>
<th>Volume of cell suspension</th>
<th>Sample volume prepared</th>
<th>Final concentration of the sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 µL</td>
<td>1000 µL of a 20 µM concentration</td>
<td>10 µL</td>
</tr>
<tr>
<td>1000 µL</td>
<td>1000 µL of a 100 µM concentration</td>
<td>50 µL</td>
</tr>
<tr>
<td>1000 µL</td>
<td>200 µL of a 1000 µM concentration</td>
<td>100 µL</td>
</tr>
<tr>
<td>100 µL</td>
<td>1000 µL of a 300 µM concentration</td>
<td>150 µL</td>
</tr>
</tbody>
</table>

Following the writing of the requirements, the prepared plate was put in a CO2 incubator for an hour. Then, trypsin and culture media that contained 25 x 104 cells per ml were made individually for each housing and utilized for the Comet procedure.

5. Comet assay

The basic objective of slide preparation is to create a gel that is homogenous and stable throughout the whole procedure and produces a comet that is visible with the least amount of background interference. The day before the experiment, the slides were prepared. A 50 ml solution of PBS was used to dissolve 500 mg of NMA. The resultant mixture was microwaved for one minute to make it clear. The cleaned slides were put in a warm, freshly made 1 percent solution to prepare them, after which they were gently and horizontally removed and set aside at room temperature. The surface underneath each slide was cleaned and agarose-free after the slides had dried. These slides were stored at room temperature and were kept dry. The strength and speed of DNA movement are constrained by high agarose concentration. Therefore, at this point, preparing slides with homogenous gel and the right concentration requires high accuracy. Slides can be kept for a few weeks in storage. After drying, slides were given labels. Three slides were created for each sample in this study (Oren et al., 2010).

To make the cell suspension, 10 ml of PBS were diluted with 0.1 g of LMA powder to make a solution with a 1 percent concentration of LMA. This solution was then microwaved for one minute to make it clear. One hundred microliters of the
contents of each falcon were transferred to the previously prepared and numbered slides after the cell suspension within each falcon had been pipetted, and 1 ml of LMA solution, which had a concentration of 1 percent at 37 °C, had been added to each falcon.

To ascertain the concentration that causes genotoxicity, the positive control slide preparation phase was carried out. At this point, all of the slides were stored in a dark, chilly setting, with the exception of the positive control slide. Two positive control slides received a sampler addition of 400 microliters of a 200 µM H2O2 solution, and the slides were then placed refrigerated for 20 minutes. Positive control slides were placed in the refrigerator and then cleaned three times for fifteen minutes with deionized water (Morgan, 2003).

After 40 minutes in the newly made cold lysing solution, all slides were removed and put in the refrigerator. The slides were rinsed three times with cold deionized water after the lysis process was complete in order to eliminate the residue of detergent salt and other contaminants from the surface of the slides. To produce single-stranded DNA and express unstable alkaline sites (ALS) in single-strand breaks, slides should be put in an alkaline electrophoresis buffer prior to electrophoresis (SSB). Slides were incubated for 40 minutes in this investigation. They were kept in the refrigerator's buffer tank. After electrophoresis, neutralizing buffer is used to remove the alkalinity of the electrophoresis environment from the slides. This buffer has 0.4 M Tris and a pH of 7.5. At this point, the slides were submerged for 10 minutes in the neutralizing buffer to remove the tank buffer’s alkaline pH.

6. Procedure for measuring the level of intracellular glutathione (GSH)

To precipitate proteins, 1.5 ml of TCA and EDTA (10%) were added to the falcon tube containing HepG2 liver cancer cells (which have been pre-treated with various doses of sodium benzoate). The samples were centrifuged at 3500 x g for 15 minutes in the following step. Once the reaction had finished for 15 minutes, 1 ml of the supernatant solution was withdrawn, 2.5 ml of buffer with a pH of 8.9 was added to it, and then 0.5 ml of DTNB (40 percent) was added to it. The tube was then well agitated until a uniform yellow hue was seen. At a wavelength of 412 nm, a spectrophotometer was used to detect absorption (Azari et al., 219).

7. The method of measuring the number of reactive oxygen species (ROS)

The HepG2 liver cancer cells and sodium benzoate were treated in accordance with the following protocol to measure the number of reactive oxygen species (ROS):

- The DCFHH-DA stock solution is made up of 10 mM in DMSO and kept at 4 °C.

- Similar to the lysing solution employed in the Comet assay, this lysing solution is utilized.
• The cells that have been exposed to the desired medicines are rinsed twice with PBS in accordance with the timing protocol.

• From the original stock solution, a working DCFH-DA solution with a final concentration of 100 M is created.

• The cells are then given 100 microliters of the DCFH-DA working solution, and they are incubated at 37 degrees for an hour.

• After the DCFH-DA solution has been withdrawn from the medium, the cells are given two further washes.

• After the cells are treated with a cold lysing solution for 1 minute, the contents of the wells are collected and centrifuged at 2800g for 5 minutes.

• The supernatant is then divided into 200 microliter portions and added to each well of a 96-well plate.

• A microplate reader (Excitation: 485 nm, Emission: 530 nm) measures the fluorescence level.

• At all times, aluminum foil and a dark environment should be utilized with the cells.

• In the test, the positive control of 0.1 mM H2O2 and the negative control of cells cultured with culture media alone were both taken into consideration (Ghassemi-Barghi et al., 2016).

The data were statistically analyzed using Prism 3 statistical software, and they are presented as mean ± standard deviation (SEM ± Mean) from three iterations of the experiment. One-way ANOVA test with Posttest: Tukey was one of the statistical tests employed, and the graph was created using the same graphic tool. The significant limit is set at P 0.05 (Ghassemi-Barghi et al., 2016).

**Findings**

1. Examining the genotoxic effect of sodium benzoate in liver cancer cell line HepG2)

The comet assay was applied in an alkaline environment to look at the genotoxicity induced by sodium benzoate. Three criteria were used to measure the outcomes of the Comet test: the tail length, the percentage of DNA in the tail, and the tail moment. These results were compared to those of the negative control group (cells that were incubated with other cells without adding any additional substances). As a positive control in this experiment, cells were positioned close to 200 M H2O2, a genotoxic substance. To verify that the experiment was conducted correctly, slides of positive and negative controls were placed adjacent to the cells that had received sodium benzoate treatment.
HepG2 cells were treated with sodium benzoate at various concentrations (150, 100, 50, and 10 M) for an hour to assess the genotoxic impact. DNA damage is depicted in graphs (1, 2 and 3) as three factors. Comparing the tail length, the percentage of DNA in the tail, and the tail moment to the control group, respectively.

For all three factors, the one-way analysis of variance (ANOVA) findings was significant. According to the analysis's findings, compared to the control group, all concentrations significantly increased the length of the tail, the percentage of DNA in the tail, and the tail moment. The symbols (*), (**), (***), and (**) denote significant differences from the control group (p<0.05), (p<0.01), (p<0.001), and (p<0.0001).

Figure 1: Tail length comparison of different concentrations of sodium benzoate in HepG2 cell culture medium

This graph compares the tail length of various sodium benzoate concentrations to the negative control group. The outcomes are shown as Mean SD based on three iterations. A significant difference from the control group is indicated by the symbol (****) (p<0.0001). The control group is made up of cells that were left alone and incubated in full culture media for 24 hours. The tail length parameter considerably increased in the HepG2 cell line at three different sodium benzoate doses (150, 100, and 50 M), indicating DNA damage.
Figure 2: Comparing the %DNA in tail resulting from Comet test of different concentrations of sodium benzoate in the culture medium of HepG2 cells

This graph compares the quantity of DNA movement at various sodium benzoate concentrations to the control group. The outcomes are shown as Mean ± SD based on three iterations. (****) denotes a noteworthy variation from the control group (p<0.0001). The control group is made up of cells that were left alone and incubated in full culture media for 24 hours. In the HepG2 cell line, the percent DNA in the tail parameter considerably increased at all four sodium benzoate doses (150, 100, 50, and 10 M), indicating DNA damage.

Figure 3: Tail moment comparison of different concentrations of sodium benzoate in the culture medium of HepG2 cells
Different sodium benzoate concentrations are contrasted with the control group in this tail moment graphic. The data is presented as Mean SD based on three iterations. A significant difference from the control group is indicated by the symbol (****) (p<0.0001). The control group is made up of cells that were left alone and incubated in full culture media for 24 hours. In the HepG2 cell line, the tail moment parameter rose dramatically at three different sodium benzoate doses (150, 100, and 50 M), indicating DNA damage.

2. Evaluating the amount of reduced intracellular glutathione caused by sodium benzoate in HepG2 cell line

HepG2 cells were treated with sodium benzoate at concentrations of 150, 100, 50, and 10 M for 1 hour in order to assess the impact of sodium benzoate on reduced glutathione levels. In contrast to the control group, graph (4) displays the average data of reduced glutathione determined by fluorimetry at wavelengths between 480 and 520 nm. Cells in the control group were cultured for a full hour with just complete culture media. All three concentrations of 150 and 100 M showed statistically significant one-way analysis of variance (ANOVA) findings. According to the analysis’s findings, a substantial drop in the quantity of reduced glutathione was seen in these three concentrations when compared to the control group. The findings are shown as Mean SEM based on three iterations. (****) denotes a noteworthy variation from the control group (p<0.0001).

3. The results obtained in evaluating the number of reactive oxygen species (ROS)

HepG2 cells were treated for 1 hour to sodium benzoate concentrations of 150, 100, 50, and 10 M to assess the impact of sodium benzoate on the number of
reactive oxygen species (ROS). In comparison to the control group, graph (5) displays the average data of the number of reactive oxygen species (ROS) determined by fluorimetry at a wavelength of 480–520 nm. Cells in the control group were cultured for a full hour with just complete culture media. All three concentrations of 150 and 100 M showed statistically significant one-way analysis of variance (ANOVA) findings. Reactive oxygen species (ROS) were found in much higher amounts in these three concentrations compared to the control group, according to the analysis’s findings. The findings are shown as Mean SEM based on three iterations. A significant difference from the control group is indicated by the symbol (****) (p<0.0001).

![Graph](image-url)

Figure 5: Determining the number of reactive oxygen species (ROS) created by sodium benzoate resulting from the Comet test in the culture medium of HepG2 cells.

**Discussion**

This study aims to investigate the genotoxicity caused by sodium benzoate in the liver cancer cell line (HepG2) by Comet assay. The findings of a study (Beezhold et al., 2014) that examined the connection between the consumption of drinks high in sodium benzoate and symptoms related to ADHD in students came to the conclusion that these data indicate that high consumption of drinks high in sodium benzoate may result in symptoms related to ADHD in students. The findings of the present study are consistent with those of the study. The toxicity of sodium benzoate on liver cancer cell lines was assessed in the current investigation. Sodium benzoate has a harmful effect on cancer cell lines, according to the findings of an investigation of its genotoxicity.

The toxicity of sodium benzoate was generated by gene alterations and changes in enzyme pathways, as in other research, and it was hazardous to zebrafish larvae (Tsay et al., 2007). The present study’s findings are consistent with this research’s findings, which indicate that sodium benzoate toxicity has been observed in liver cancer cells.
According to a study, free radicals are produced by oxidative stress, which increases the risk of chronic disease and damages healthy cells (Yetuk et al., 2014). Additionally, the genotoxic effects of sodium benzoate include chromosomal breaks, the development of micronuclei, and mutations in the Ala40Thr superoxide dismutase gene (Pongsavee, 2015). The findings of this study are compatible with those of the current study since superoxide dismutase is an enzyme that repairs cells and eliminates superoxide, the most prevalent free radical in the body. Different sodium benzoate doses were shown to enhance reactive oxygen species while decreasing reduced glutathione in this investigation. As a result, it generates oxidative stress, which results in the generation of toxic effects and damage to the DNA strand by upsetting the equilibrium between active species and the body’s antioxidant system in favor of active species and free radicals.

In research, sodium benzoate was used to control plasma glycine levels in five kids with non-ketotic hyperglycemia in infancy. This approach reduced seizures and markedly increased alertness. Patients’ quality of life was enhanced by high doses of sodium benzoate (Van Hove et al., 1995). The present study was conducted at concentrations of 10, 50, 100, and 150 µM, and the results of the evaluation in these concentrations showed that the effect of toxicity was observed in three concentrations of 50, 100, and 150 µM, while in the concentration of 10 µM, no significant difference was observed. It is possible that the results of this study are inconsistent with the results of the present study. According to the findings, DNA was significantly damaged by sodium benzoate when compared to the control group. Additionally, sodium benzoate’s genotoxicity is concentration-dependent. The liver cancer cell line’s reactive oxygen species (ROS) levels were found to be considerably higher in the three concentrations of sodium benzoate tested—150, 100, and 50 µM—than in the control group (HepG2).

Conclusion

Liver cancer cells are toxic to sodium benzoate (HepG2). According to the findings, DNA was significantly damaged by sodium benzoate when compared to the control group. Since sodium benzoate’s genotoxicity is concentration-dependent, liver cancer cell lines exposed to sodium benzoate at greater concentrations than the control group had an increase in reactive oxygen species (ROS) (HepG2).

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