SODIUM BENZOATE IN LOCALLY AVAILABLE SOFT DRINKS AND ITS EFFECT ON DNA DAMAGE AND LIVER FUNCTION IN RATS

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Abstract

Sodium benzoate (E211) is used as a preservative in several kinds of food. One of the essential uses of E211 is to preserve non-alcoholic carbonated beverages. The amount of sodium benzoate in 17 local soft drink samples was estimated in the present study. The *in vivo* effects on biochemical aspects of the liver, kidney function, and DNA damage in lymphocytes were also investigated after oral administration of sodium benzoate in rats over 12 days. The control and experimental groups were fed standard pellet diet and distilled water *ad libitum;* and administered distilled water (control) and different concentrations (30, 60, and 120 mg/kg body weight) of sodium benzoate once daily through a stomach tube (0.5 ml), respectively. The alkaline comet assay was performed to investigate the possible DNA damage induced by E211 in lymphocytes. At the end of the experiment, after overnight fasting the rats were sacrificed and blood samples were collected. Animals showed no significant weight loss or gain. The investigation revealed that twelve samples contained a higher level, and the rest had trace or below the recommended maximum permitted concentration (150 mg/l) of E211 as a preservative in the soft drinks. The percentage of tail DNA ($p < 0.01$) and olive tail movement ($p < 0.05$) was significantly increased in lymphocytes that were treated with high concentration (120 mg/kg body weight) of sodium benzoate, indicated substantially higher DNA damage (3.5 times) in lymphocytes compared to control. Moreover, serum AST activity level was increased significantly ($p<0.05$) in the group treated with a higher dose (120 mg/kg body weight) of E211, indicating malfunction of the liver. The preservative did not significantly affect serum ALT and ALP activity, total bilirubin, creatinine, and urea level. Nonetheless, our findings suggest that caution should be adopted for using sodium benzoate as a preservative in various food products.

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Introduction

Sodium benzoate (E211) is used as a preservative for inhibiting molds, yeasts, and bacterial growth in various products such as pickles, sauces, marmalades, margarine, beverages, and juices[1]. Although used in several foods, soft drinks and juices are the primary dietary sources of this preservative. According to European Union Legislation, the maximum permitted quantities of sodium benzoate in non-alcoholic soft drinks allowed was established to 150 mg/l[2]. The Expert Committee on Food Additives (JECFA) of Joint Food and Agriculture Organization (FAO)[3] of the United Nations and World Health Organization (WHO) as well as WHO[4] International Programme on Chemical Safety (IPCS) considers daily intake of 5 mg/kg body weight of sodium benzoate to be safe for human consumption. Even though sodium benzoate is permitted to be used as a food preservative, there is still continuous debate and controversy over its effects on human health. It may cause severe health problems for people who consume high sodium benzoate for a long time. This is because in our body, in the presence of vitamin C, sodium benzoate is converted to benzene[5]. This well-known carcinogen can cause a single strand or double-strand breakage in nuclear DNA[6].

The toxic effects of sodium benzoate have been studying by many researchers, and they have suggested adverse effects due to the intake of sodium benzoate. Studies have suggested that sodium benzoate caused considerable hepatocellular damage, induced swelling (vacuolization), deterioration of chromatin material, disorganization of hepatocytes, dilated central vein, hemorrhage, and syncytium formation in the liver of animals[7-10]. Alteration in hepatic and renal function with oral administration of a subchronic dose of sodium benzoate was also reported by Oyewole *et al.*[11]. Several studies reported that sodium benzoate caused histological alterations in the liver and kidney tissues[12,13] and induced renal toxicity in the rat[14]. Ibekwe *et al.*[15] reported that the plasma level of aspartate aminotransferase (AST), a marker enzyme for liver function, was increased in the short-term treatment of experimental animals with sodium benzoate. Further, Ahmad *et al.*[16] reported significantly increased liver function tests (ALT, AST, and bilirubin) and kidney function tests (urea and creatinine) in sodium benzoate exposed rats with doses and length of the study period. Helal *et al.*[17] and Tawfek *et al.*[18] also reported significantly higher values of these parameters in response to different sodium benzoate treatments. Fujitani[19] reported that short-term single toxic dose treatment did not show any significant change in serum AST and ALT activity level in B6C3F1 mice and F344 rats compared to the control group.

There is increasing evidence suggesting that a high intake of sodium benzoate would be associated with hyperactivity and attention-deficit/hyperactivity disorder (ADHD) in young children[20-23]. In the behavioral tests, sodium benzoate treatment also showed learning and memory deficits in mice[24] and motor impairment in rats[25]. Yavav *et al.*[26] suggested the immunomodulatory potential of sodium benzoate when consumed above

the acceptable daily intake. On the other hand, the adverse effects of sodium benzoate intake were not found by other reports[27-29].

However, little has been published related to the effect of sodium benzoate on DNA damage. Zengin *et al.*[30] reported that sodium benzoate decreased mitotic index and increased chromosomal aberrations, sister chromatid exchange, micronucleus (MN) frequency, and significantly increased nuclear DNA damage in human lymphocytes assessed by comet assay. Others also reported genotoxic and cytotoxic effects of sodium benzoate in lymphocytes caused by a significantly increased level of chromosomal aberration, sister chromatid exchanges, and micronucleus formation[31,32]. On the other hand, Sasaki *et al.*[33] reported that sodium benzoate (2000 mg/kg) did not cause any significant DNA damage in mice organs. Studies also reported that sodium benzoate has no adverse carcinogenicity and genotoxicity effect in human lymphocytes[34,35].

Sodium benzoate, which has been reported to cause a severe adverse effect, is widely consumed by the human population as food additives[36]. Thus, the biosafety of sodium benzoate is a critical issue that should be addressed. Therefore, this study was undertaken to estimate the amount of sodium benzoate present in various soft drinks available in local markets of Bangladesh and to study the possible short-term effects of sodium benzoate on DNA damage and a dose-dependent impact on liver and kidney function in the rat.

Materials and Methods

Collection of soft drinks: Seventeen locally available and widely consumed pre-packaged non-alcoholic carbonated beverages (soft drinks) were collected from Dhaka's local markets. Among them, seven were lemon-flavored drinks, three were cola-flavored drinks, three were orange-flavored drinks, three were energy drinks, and one was diet drinks.

Chemicals and reagents: HPLC grade acetonitrile and anhydrous sodium benzoate standard were purchased from Sigma-Aldrich Inc., Germany. Glacial acetic acid and sodium acetate were purchased from Merck, Germany, and all other reagents were analytical grade. Milli-Q water was used to prepare the solutions and mobile phases.

Preparation of the sample and sodium benzoate standard: Soft drinks of different brands and batches were purchased from the local markets of Dhaka city. Three batches (5-10 mL thoroughly mixed portions) of each brand were homogenized and immediately de-gasified in an ultrasound bath. Standard sodium benzoate solution was prepared by dissolving different concentrations of sodium benzoate in Milli-Q water. All samples were filtered through a 0.45 μm pore-sized filter (Millipore) before injected into the chromatograph. All investigated soft drink samples were analyzed directly without any further preparation steps such as extraction or concentration.

HPLC analysis: The concentration of sodium benzoate in a soft drink was measured by HPLC, following a slightly modified Pylypiw and Grether[37] method. In brief, the HPLC apparatus (Shimadzu LC-20 AT, Japan) was equipped with an SCL-10Avp system and two LC-20AT pumps, an in-line degasser, an auto sampler (30 µl injection volume), a reverse-phase column (Nucleosil C18, 5 µm, 4.6x250 mm) and a UV-VIS detector (Shimadzu SPD-20A). The experiments were performed at ambient temperature and under an isocratic solvent system using a mobile phase composed of a mixture of acetonitrile and an aqueous acetate buffer of pH 4.4 (20:80 v/v), at a flow rate of 1.25 ml/ min. The chromatograms were recorded at 254 nm. Data for each sample and standard were acquired and processed using LC solution software (Version 1.03 SP3, Shimadzu, USA) running under Windows XP on a Pentium PC.

The concentration of sodium benzoate in the sample (soft drink) was calculated by: Sodium benzoate (mg/L) = C x (PHsa/PHst) x (Vst/Vsa) x 1000, where C = concentration of standard in mg/mL, PHsa and PHst = average peak heights of sample and standard respectively, Vsa and Vst = volume injected in µl of sample and standard, respectively. Each value corresponded average of three results of a soft drink brand.

Animals: Twelve Long-Evans male rats (~ 11 weeks, the average weight of 179 g) were selected from the animal house facility of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). The rats were kept at a constant room temperature of 22 ± 5^{0}C with humidity of 40-70% and 12 hours day-night cycle.

Experimental design: The rats were divided into four groups (3 rats each) and kept individually in wire cages in the BIRDEM animal house. All rats' initial body weights were measured before starting the experiment and the rats were distributed in different groups of almost equal mean weight. Both control and experimental rats were fed standard laboratory pellet diet and water *ad libitum*; administered different concentrations (30, 60, and 120 mg/kg body weight) of sodium benzoate (0.5 ml) and water (control) daily through a stomach tube for 12 days, respectively. The body weights of animals were also measured on the 5th and 12th day to determine the body weight changes during the experiment. At the end of the experiment, the rats were sacrificed after overnight fasting, and blood samples were collected for comet assay and measuring different serum parameters. The study was conducted in BIRDEM, according to the approved institutional ethical guideline for the use and care of laboratory animals.

Collection of the blood sample: The blood sample was collected from the abdominal aorta in a disposable plastic syringe in falcon tubes (4-5 ml) and EDTA-containing tubes (2 ml). The samples in EDTA-containing tubes were immediately transported to the laboratory in an ice pack container and kept at -80°C for DNA damage analysis. The falcon tubes were centrifuged at 3,000 rpm for 15 min; the serum was separated and stored at -20ºC until further investigation.

Evaluation of DNA damage by comet assay: The possible DNA damage induced by sodium benzoate in lymphocytes was measured by using the alkaline comet assay. The comet assay was carried out by a slightly modified method of Tice *et al.*[38]. Lymphocyte was suspended in 75 µl 0.7% low melting point agarose in PBS at 37ºC and placed on a microscopic slide with a 1% agarose layer. For DNA unwinding, the slides were immersed in lysis solution at 4ºC for 1 hour and then placed in a single row in a horizontal electrophoresis tank containing alkaline buffer at 4ºC for 20 minutes. Then, at the same temperature, the electrophoresis was carried out at 25V, 300 mA for 40 minutes. The slides were washed with a neutralization buffer three times (5 minutes each) after removing the slides from the lysis solution and then visualized after silver staining according to the method of Nadin *et al.*[39]. All sample preparation steps for electrophoresis were conducted under yellow light to minimize the possibility of damage to cellular DNA. The comet images of both control and sodium benzoate treated animal's lymphocytes were analyzed microscopically using the software Comet Assay Software Project (CASP, version 1.2.2), established by Konca *et al.*[40]. The threshold values of CASP parameters were adjusted to achieve the optimum values for our staining procedure; DNA damage was quantified by calculating the comet parameter: percentage of DNA in the comet's tail (%TD) and olive tail moment (OTM). The Olive tail moment is the percent of DNA in the tail multiplied by the distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head in the x-direction. The more severe the damage, the higher the percent of DNA fragmentation. Similarly, the longer the comet tails, the higher the DNA fragmentation and the more severe the damage. Thus, the higher the values of %TD and OTM, the higher the DNA damage[41].

Biochemical analysis: Standard laboratory methods estimated aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities, and total bilirubin, creatinine, urea content in serum by using commercial kits.

Statistical analysis: All data were analyzed with the statistical program GraphPad Prism 5. One-way analysis of variance (ANOVA) was used, followed by the Bonferroni test. The results are presented as mean±SEM. The $p < 0.05$ was considered statistically significant.

Results and Discussion

Sodium benzoate is widely used in the food industry as a preservative, especially in soft drinks. Table 1 shows the concentration of sodium benzoate. Out of 17 soft drink samples, 12 contained a higher level of sodium benzoate as a preservative than that of the maximum permitted limit, which is 150 mg/l for non-alcoholic soft drinks. Only five samples contained trace or below the maximum allowed level of sodium benzoate. The average sodium benzoate concentration used as a preservative in soft drinks ranged from 2.1 to 235.9 mg/L (Table 1). Similarly, Khosrokhavar *et al.*[42] reported high levels of

sodium benzoate in soft drinks available in Iran, higher than maximum acceptable limits specified by national standards. On the other hand, Alghamdi *et al.*[43] reported the content of sodium benzoate in soft drinks available in Riyadh city maintained the acceptable range authorized by Saudi Arabian food regulations authority.

Table 1. Sodium Benzoate Contents in Soft Drinks.

Sample Number	Sodium benzoate (mg/L)	Above/Below of maximum permitted value*
1.	108.5 ± 1.9	↓
2.	2.1 ± 0.03	Trace
3.	2.6 ± 0.003	Trace
4.	155.3 ± 0.2	↑
5.	235.9 ± 3.7	↑↑↑
6.	200.2 ± 2.7	↑↑↑
7.	158.7 ± 0.6	↑
8.	138.3 ± 0.8	↓
9.	155.7 ± 1.5	↑
10.	199.2 ± 1.95	↑↑
11.	127.3 ± 1.03	↓
12.	165.7 ± 1.3	↑
13.	175.4 ± 0.97	↑↑
14.	164.9 ± 0.99	↑
15.	197.9 ± 0.3	↑↑
16.	219.5 ± 2.03	↑↑↑
17.	203.3 ± 2.9	↑↑↑

Values are mean ± SEM. Each value represents the triplicate measurement.
*Maximum permitted value: 150 mg/L (European Parliament & Council
Directive No 96/77/EC, 1996 and Commission Directive No 2006/12/EC, 2006).

Figure 1 shows the group-specific sodium benzoate concentration in soft drinks. When the samples were presented in 5 different groups according to flavor type, it was found that cola-flavored drinks contained lower than the acceptable sodium benzoate content. The other four groups were provided above the maximum permitted limit. Among the three cola-flavored drinks, two samples contained a trace amount (mean value: 2.1 mg/L and 2.6 mg/L). Another sample included a mean value of 108.5 mg/L, much below than that of the maximum permitted level of sodium benzoate. Fig. 1 shows that the energy drink contained the highest sodium benzoate concentration and much higher (206.89±3.4 mg/L) than that of the permitted value. One possible reason for using a higher amount of preservative in energy drinks may be to prevent the natural growth of microorganisms in energy drinks, which usually contain a much higher sugar level.

These findings advocate that soft drinks commercially available in Bangladesh pose considerable public health risks.
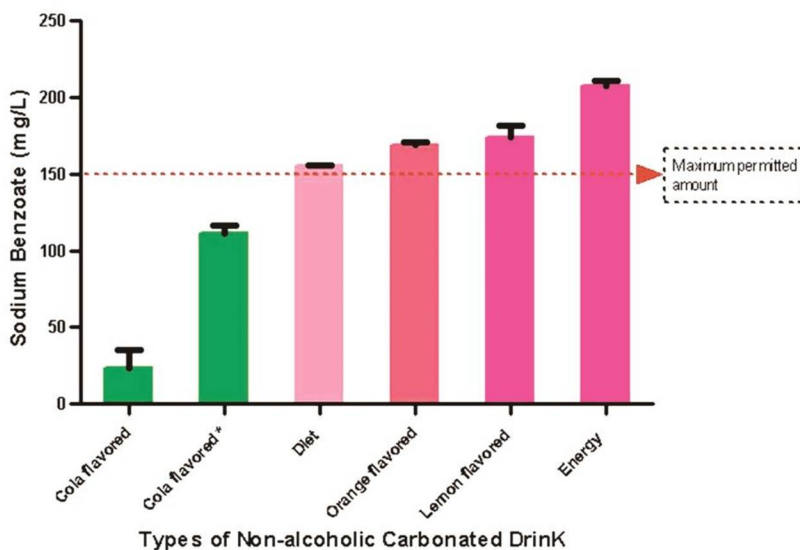

Fig. 1. Group-specific concentration of sodium benzoate in soft drinks.
*Sample #1 only. Each type represents the triplicate measurement of each sample.

In this study, the effect of sodium benzoate on DNA damage in rat lymphocytes by alkaline comet assay or single cell gel electrophoresis was evaluated. The percent of DNA present in the tail region can determine the DNA damage level in comet analysis. As shown in Fig. 2A, there was little or no evidence for comet formation for control rats, maintaining a normal circular nucleus with tightly compressed DNA. In contrast, lymphocytes from rats treated with different doses of sodium benzoate for the short term (12 days) showed abnormal appearance having an extended tail nucleus (Fig. 2B-D) due to DNA fragmentation. This data indicates that comet tail length increased in dose-dependent manners with sodium benzoate in different rat groups. The migration of damaged head DNA from the head to the tail region was taken place during electrophoresis. The most extended tail was found in the 120 mg/kg group (Fig. 2D). The length and intensity of the comet tail relative to the head reflect the number of DNA breaks. Cells containing higher levels of DNA strand breakage create comets with more intense 'tails' (Fig. 2D). Short-term (12 days) administration of sodium benzoate had no effects on body weight (data not shown). This finding is consistent with a previous study[12] but contrary to Priya et al.[44]. They reported a dose and time-related significant body weight gain in rats treated with sodium benzoate compared to the control group.
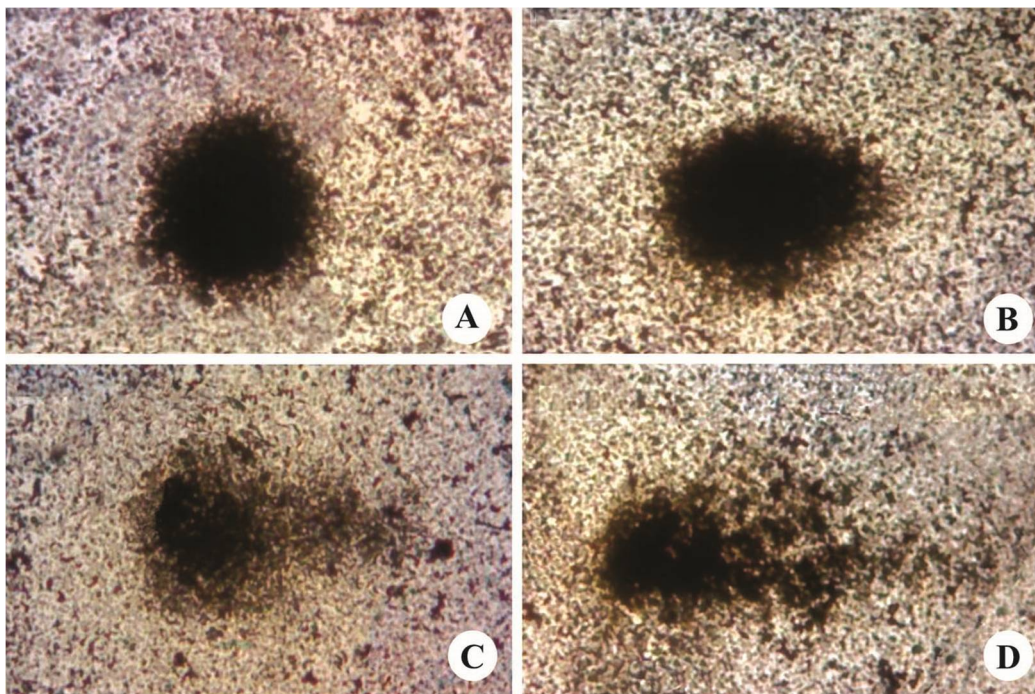
Fig. 2. Comet images of lymphocytes of control and sodium benzoate treated rats. Following 12 days treatment with 0 (A), 30 (B), 60 (C) and 120 (D) mg/kg BW sodium benzoate, the extend of DNA damage was assessed.

The mean values of comet parameters are presented in Fig. 3. As shown in Fig. 3, the dose-dependent increase in tail DNA has been observed following treatment with 30, 60, and 120 mg/kg of sodium benzoate, respectively, compared to controls. The percentage of tail DNA (p<0.01) and olive tail movement (p<0.05) was significantly increased in lymphocytes that were treated with high concentration (120 mg/kg body weight) of sodium benzoate compared to the control group. A high dose of sodium benzoate (120 mg/kg body weight) induced significantly higher DNA damage (3.5 times) in lymphocytes than in control. Similar to our study, Zengin *et al.*[30] reported significantly increased DNA damage by sodium benzoate in human peripheral blood lymphocytes. The genotoxic, cytotoxic, and mutagenic effects of sodium benzoate on human lymphocytes were also reported by Petel and Ramani[31] and Pongsavee[32].

In this short-term study, the level of serum aspartate aminotransferase (AST) activity increased in all groups of rats, but only significantly (p < 0.05) in 120 mg/kg body weight administered rats (Fig. 4), indicating a possible functional disturbance in the liver. Ibekwe *et al.*[15] also reported an increased AST level in plasma after short-term treatment of rats with sodium benzoate. On the other hand, Fujitani[19] reported that short-term

single toxic dose treatment did not significantly change serum AST and ALT activity level in B6C3F1 mice and F344 rats compared to the control group. In the present study, although the ALT level was also increased in all sodium benzoate treated groups compared to control, it was not statistically significant (data not shown). No significant
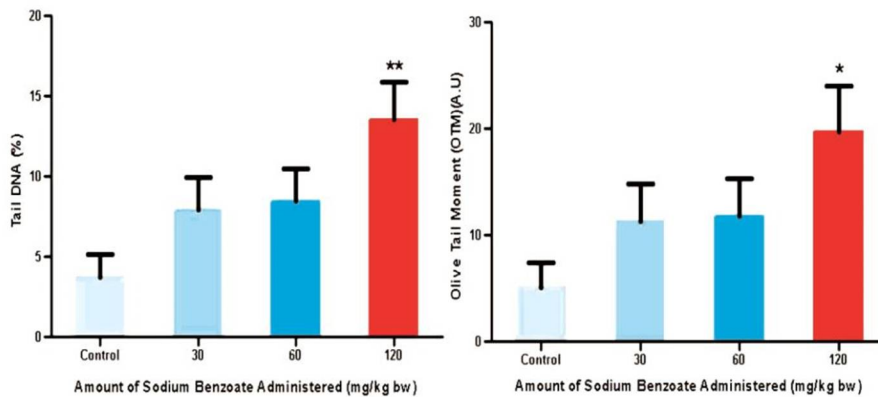

Fig. 3. Tail DNA (%) and Olive Tail Moment in Lymphocytes of Control and Sodium Benzoate Treated Rats. Each value represents the mean±SEM. **p < 0.01 and *p < 0.05 significantly different from control. Tail DNA (%) and Olive tail moment were determined in ~100 cells per animal. The olive tail moment is expressed in an arbitrary unit (A.U.).
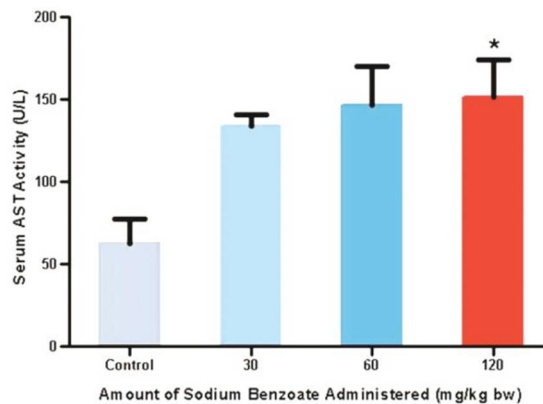

Fig. 4. Serum Aspartate Aminotransferase (AST) activity in control and Sodium Benzoate Treated Rats. Each value represents the mean±SEM. *p< 0.05 significantly different from control.

difference was found in serum ALP activity, total bilirubin, creatinine, and urea level of sodium benzoate treated rats than control (data not shown). Hassan et al.[8] also reported a significant gradual increase (according to increased benzoic acid treatment doses) in serum ALT in treated animals compared with control.

In contrast to our findings, significant elevation of serum ALT, urea, uric acid, and creatinine was reported by Oyewole[11] in sodium benzoate treated rats. Further, Ahmad *et al.*[16] reported significantly increased ALT levels, AST, bilirubin, urea, and creatinine in the blood of sodium benzoate exposed rats depending on the dose and duration of the study. Helal *et al.*[17] and Tawfek *et al.*[18] also reported significantly higher values of these parameters in response to different sodium benzoate treatments. Notably, the experimental period of the present study was much shorter, and the concentrations of sodium benzoate used were much lower than that of previous reports[11,16-18].

Based on the present study results, it may be concluded that consumption of soft drinks containing a high concentration of sodium benzoate for a long time may cause deteriorating effects on human health, especially children. Therefore, caution is needed to use sodium benzoate as a preservative in various products consumed by humans. Further research is necessary to examine the effect of sodium benzoate on human health.

Acknowledgment

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