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Cytotoxic and genotoxic effects of sodium hypochlorite on human peripheral lymphocytes in vitro

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Abstract Chlorination is widely used method in the disinfection of drinking and utility water worldwide. In this study, cytotoxic and genotoxic effects of sodium hypochlorite were investigated by the cytokinesis-block micronucleus assay and chromosomal aberration analysis on human peripheral lymphocytes in vitro. A significant increase in chromosomal aberration frequency was observed in all treatments of NaOCl (0.030, 0.065, 0.100, 0.25, 0.5, 1, 2, 4 μg/mL) at 24 and 48 h compared with the negative control and mitomycin C (MMC, 0.3 μg/mL), which was used as a positive control. NaOCl significantly increased the frequency of micronuclei in a dose dependent manner. The results showed that there was a significant correlation between NaOCl concentration and chromosomal aberration, micronuclei frequency, necrotic cells, apoptotic cells and binucleated cells.

Keywords Sodium hypochlorite · Chromosomal aberrations (CA) · Micronucleus (MN) assay · Necrotic cell · Apoptotic cell

Introduction

Chlorine is produced for use as a disinfectant and bleach for both domestic and industrial purposes, and is also widely used to disinfect drinking water and swimming pool water and to control bacteria and odours in the food industry. A dilute solution has very stable hypochlorite ions and is a strong chemical oxidant, and free chlorine does not occur in nature. Most disinfected drinking water contains chlorine at concentrations of 0.2–1 mg/L (White 1978). Cake flour bleached with chlorine contains chlorine at levels in the range 1.3–1.9 g/kg. Unbleached flour may contain small amounts of chlorine (400–500 mg/kg). Sodium hypochlorite has a wide range of antimicrobial activity. Generally, viruses and vegetative bacteria are more susceptible to hypochlorites than endospore-forming bacteria, fungi, and protozoa (Rutala and Weber 1997). Dental root canal procedures are commonly performed to save diseased teeth. Sodium hypochlorite is commonly used as a root canal irrigant to disinfect the canal prior to filling and placement of a cap (Bystrom and Sundqvist 1983). Though sodium hypochlorite is a widely used substance for disinfection, when it is added to water or waste water, the solution easily reacts with organic material, for example humic and fulvic acids in surface water, causing the generation of many volatile and non-volatile disinfection by-products toxic for the aquatic environment (Emmanuel et al. 2004) and with mutagenic and/or carcinogenic activity on human and aquatic organisms (Monarca et al. 2000; Guzzella et al. 2004; Crebelli et al. 2005). These organic halogenated compounds include haloalkenes, haloacetic acids, haloacetonitriles, haloketones and haloaldehydes,

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bromoform, dichloroacetic acid, dibromoacetic acid, 5-methyl-2-furancarboxyaldehyde, 3-acetyl-dihydro-2(3H)-furanone (Monarca et al. 2004). Genotoxic activity and carcinogenic action in chlorinated drinking water are also considered to come from OCl-itself (Ohnishi et al. 2002; Whiteman et al. 1997). Indeed, NaOCl is considered non-classifiable for its carcinogenicity by IARC (1991) although it was shown to give positive results in some short-term mutagenicity tests. Sodium hypochlorite has been found to be mutagenic in *Salmonella typhimurium* TA1530 and TA100 but not TA1538 (Wlodkowski and Rosenkranz 1975; Ishidate et al. 1984). On the other hand, Le Curieux et al. (1993) did not detect any significant mutagenic activity on TA100, TA102, and TA98 strains. In mammals, sodium hypochlorite produced chromosomal aberrations in Chinese hamster fibroblast cells without metabolic activation (Ishidate et al. 1984). Hypochlorite ions and hypochlorous acid were negative in the in vivo erythrocyte micronucleus assay and in bone marrow aberration studies in mice (Meier et al. 1985). Buschini et al. (2004) reported sodium hypochlorite effects on *Saccharomyces cerevisiae* without endogenous metabolic activation whereas a clastogenic effect was detected by the newt micronucleus assay, a sensitive method for testing promutagens (Le Curieux et al. 1993).

Since the data on sodium hypochlorite biological effects are partially conflicting, further studies have to be performed to clarify the toxic/genotoxic potency of this compound. In this context, the present work focuses on the in vitro analysis of cytotoxic and genotoxic effects of sodium hypochlorite, using cytogenetic tests such as the cytokinesis-block micronucleus assay and chromosomal aberration analysis in human lymphocytes.

Materials and methods

Human peripheral blood cells were used as the test system. Peripheral venous blood was collected from healthy non-smoking adult males and females aged 18–22 years. Donors provided written, informed consent at the time of donation for the use of their blood sample in this study. Whole blood (0.4 mL) was added to 5 mL chromosome medium (Euroclone). NaOCl was added after 24 and 48 h of culture initiation. Human lymphocytes were exposed to different concentrations (0.015, 0.030, 0.065, 0.100, 0.25, 0.5, 1, 2, 4 μg/mL) of NaOCl. A negative (distilled water 1%) and a positive control (mitomycin-C (MMC), 0.3 μg/mL) were included in every experiment (Roncada et al. 2004). Colchicine (0.06 μg/mL) was added to all the cultures at 70 h. The cells were collected by centrifugation (377 × *g*, 10 min), and resuspended in a hypotonic KCl solution (0.4%) for 30 min at 37 °C. Following this process, the cells were centrifuged again and fixed in a cold methanol:acetic acid (3:1) mixture for 35 min at +4 °C. At the end of this procedure, the cells were treated with fixative two times. Then slides were made by dropping the concentrated cell suspension and air drying. Air-dried slides were stained for 15–20 min with 5% Giemsa stain (pH 6.8) prepared in Sorensen buffer. One hundred metaphases per culture were analysed for the presence of chromosomal aberrations (CA). The number of CA was obtained by calculating the percentage of metaphases at each concentration and treatment period that showed structural and numerical chromosome aberrations. Chromatid and chromosome breaks, chromosome exchange and chromatid union, and polyploid cells were screened in all treatment concentrations. (Rencuzogullari and Topaktas 2000; Yuzbasioglu et al. 2006).

For the cytokinesis-block micronucleus assay (CBMN), the blood samples were added to 5 mL chromosome medium (Euroclone). The cultures were incubated at 37 °C for 72 h. Twenty-eight hours before harvest, cytochalasin-B (6 μg/mL) was added to each culture. Human lymphocytes were exposed to different concentrations (0.5, 1, 1.5, 2, 2.5 μg/mL) of NaOCl for the MN test. The cells were exposed to the chemical after 24 and 48 h of culture initiation. The cells were harvested by centrifugation (167 × *g*, 10 min), and the pellets were resuspended in a hypotonic solution of 0.075 M KCl for 5 min at +4 °C. The cells were again centrifuged and fixed in the cold methanol:acetic acid (3:1) mixture for 15 min. The fixation procedure was applied three times. Formaldehyde (1%) was added to the last fixative to preserve the cytoplasm. The slides were made by dropping the concentrated cell suspension and air drying. For the MN analysis, staining was done with 5% Giemsa (pH = 6.8), prepared in Sorensen buffer solution, for 20–25 min. The slides were then washed in distilled water, and dried at room temperature. Mitomycin-C (MMC), 0.50 μg/mL

Table 1 Chromosome aberrations produced by NaOCl in human lymphocytes in vitro

Test substance	Treatment		Structural aberrations				Numerical aberrations	Frequency of aberrant cell ± SEM (%)
	Period (h)	Doses (μg/mL)	ctb	csb	cse	cu	*p*	
NC	24	1	1	–	–	–	–	0.2 ± 0.2
MMC	24	0.3	19	2	3	7	1	6.2 ± 3.3
NaOCl	24	0.015	1	–	–	–	–	0.2 ± 0.2
		0.030	7	1	1	1	1	2.2 ± 1.2*
		0.060	8	1	4	1	1	2.8 ± 1.4*
		0.100	9	1	2	–	–	2.4 ± 1.6*
		0.25	13	2	4	2	–	4.2 ± 2.7*
		0.5	15	3	1	1	1	4.0 ± 2.7*
		1	14	1	1	0	1	3.2 ± 2.6*
		2	15	2	2	2	1	4.2 ± 2.7*
		4	13	2	4	2	2	4.2 ± 2.2*
NC	48	1	1	–	–	–	–	0.2 ± 0.2
MMC	48	0.3	24	3	2	5	2	6.8 ± 4.3
NaOCl	48	0.015	–		1	–	–	0.2 ± 0.2
		0.030	7	1	2	–	1	2.0 ± 1.2*
		0.060	10	1	2	1	1	2.8 ± 1.8*
		0.100	10	2	1	1	1	2.8 ± 1.8*
		0.25	14	2	4	3	2	4.6 ± 2.4*
		0.5	12	4	1	1	1	3.6 ± 2.1*
		1	15	1	1	1	2	3.6 ± 2.8*
		2	13	1	2	1	3	3.4 ± 2.4*
		4	16	3	3	2	2	4.8 ± 2.8*

ctb chromatid break, *csb* chromosome break, *cu* chromatid union, *cse* chromosome exchange, *nc* negative control (1% distilled water), *MMC* (0.3 μg/mL mitomycine-C (24, 48 h)

* $P < 0.05$ as compared to control. *Fisher's* exact test

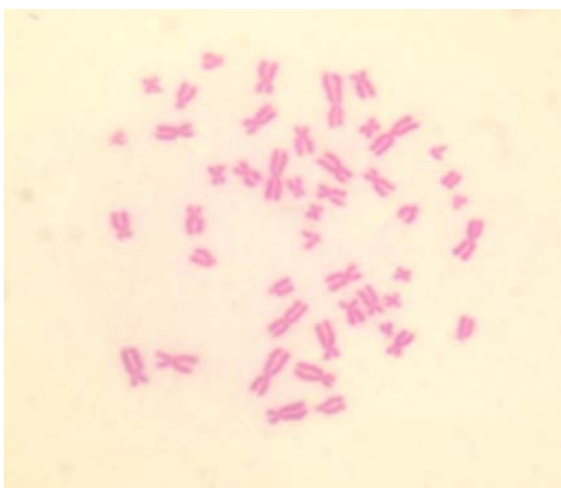

Fig. 1 Normal chromosomes obtained from healthy non-smoking adults

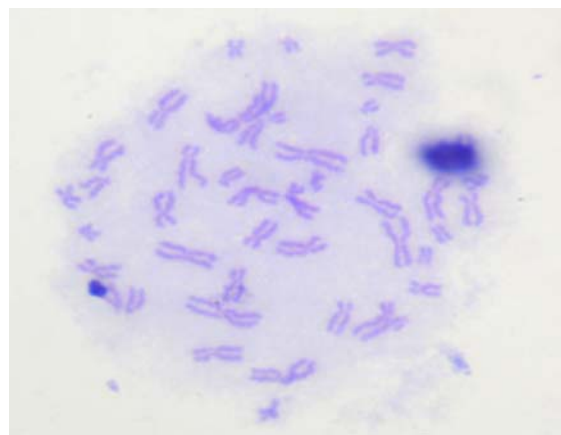

Fig. 2 Effects of mitomycin-C 0.3 μg/mL (positive control) on human lymphocyte chromosomes

(Demsia et al. 2007), was used as a positive control. In accordance with standard criteria (Fenech et al. 1999), MN analysis was performed on coded slides by scoring 2,000 binucleate lymphocytes for each subject. The nuclear division index (NDI) was calculated following the formula: NDI = $[M_1 + 2(M_2) + 3(M_3) + 4(M_4)]/N$, where M_1–M_4 indicates the number of cells with 1–4 nuclei and N indicates the total number of cells scored. The NDI of each cytochalasin B-treated culture was determined by screening 2,000 interphase cells for the number of nuclei included (Eastmond and Tucker 1989).

Apoptotic and necrotic cells were identified by light microscopy using morphological characteristics of the nucleus. In order to distinguish apoptotic cells from necrotic cells, we checked the properties of both cells, which exhibit a pale cytoplasm or a loss of cytoplasm, numerous vacuoles, and a damaged/irregular nuclear membrane with a partially intact nuclear structure in necrotic cells while apoptotic cells show chromatin condensation with intact cytoplasmic and nuclear boundaries as well as cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane (Fenech et al. 1999). A total of 2,000 cells were counted for each sample. The nuclear division cytotoxicity index (NDCI) = $[A_p + Nec + M_1 + 2(M_2) + 3(M_3) + 4(M_4)]/N$ was calculated according to Fenech et al. (1999), where A_p = number of apoptotic cells, Nec = number of necrotic cells, M_1–M_4 = number of viable cells with 1–4 nuclei and N = total number of cells scored.

All statistical analyses were performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego California USA). The frequency of chromosomal aberration in cell cultures was analysed with *Fisher's* exact test. MN data were statistically analysed with the *F*-test for analysis of variance (ANOVA) and the significance of differences between the negative control and a series of treated groups was determined with Dunnett's *t*-test.

Results and discussion

Two parameters (CA and CBMN) were used to evaluate the genotoxic effects of NaOCl on human

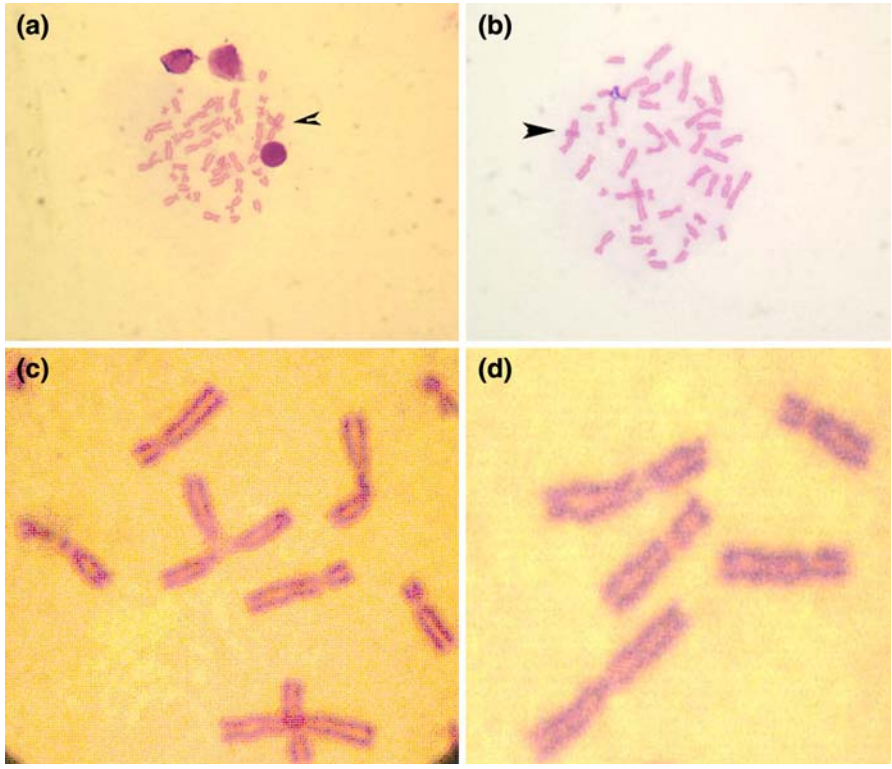

Fig. 3 NaOCl causes: **a**, **b**, **c** Chromosome exchanges (*arrows*). **d** Chromatid unions and chromatid breaks

lymphocyte cells. A significant increase in chromosomal aberration frequency was observed in all treatments of NaOCl (0.030, 0.065, 0.100, 0.25, 0.5, 1, 2, 4 μg/mL) at 24 and 48 h compared with the negative control and mitomycin C (MMC, 0.3 μg/mL), which was used as a positive control (Table 1). Normal chromosomes obtained from healthy non-smoking adults are shown in Fig. 1. Examination of lymphocyte chromosomes revealed chromosome exchange and chromatid union (Figs. 2, 3a–d), and chromatid and chromosome breaks (Fig. 4a, b) in the positive control (Fig. 2) and all treatment concentrations except for 0.015 μg/mL. Some NaOCl concentrations (0.030, 0.065, 0.100, 1, 2, 4 μg/mL) and the positive control produced polyploidy.

Statistical analysis demonstrated that significant increases in CA frequency after the addition of NaOCl concentration after 24 and 48 h ($r = 0.95$), compared with the negative control, in all treatment groups in a dose dependent manner ($r = 0.85$). The mitomycin treatment also caused a significant increase ($P < 0.001$) in the frequency of aberrant cells. Treatment with 0.05 μg/mL NaOCl did not increase the CA frequency in human lymphocyte cells.

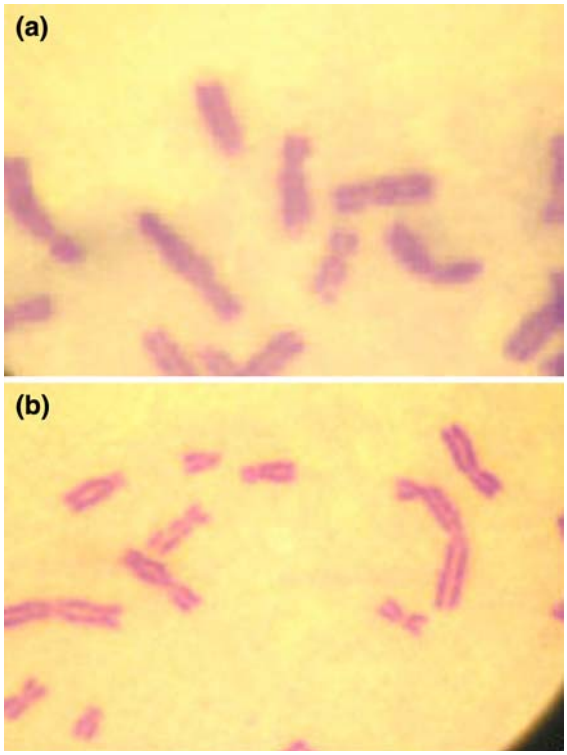

Fig. 4 All treatments concentrations of NaOCl and positive control (MMC) causes chromosomal break in human lymphocyte chromosomes

NaOCl significantly increased the frequency of micronuclei in a dose dependent manner ($r = 0.68$). Table 2 demonstrates that the nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI) were significantly influenced by NaOCl. NDI and NDCI values were lower than in the control. The lowest NDI values were observed in 1 μg/mL NaOCl-treated cultures. We demonstrated that the presence of a micronucleus in binucleated cells correlates with the induction of apoptosis ($r = 0.93$) and necrosis ($r = 0.72$).

The results showed that there was a significant correlation between NaOCl concentration and chromosomal aberration, micronuclei (Fig. 5a) frequency, apoptotic (Fig. 5b) cells necrotic (Fig. 5c) cells, and binucleated cells (Table 2).

Contaminated water causes epidemic diseases like typhoid fever, hepatitis A, polio and cholera as well as diarrhoea. To prevent these kinds of water-based disease, chlorine has been added to water for almost 100 years and chlorine is present in most disinfected drinking water at concentrations of 0.2–1 mg/L.

Studies show that drinking chlorinated water greatly affects the proliferation and growth system besides resulting in urinary and gastric cancer (Guzzella et al. 2004). Gustavino et al. (2005) showed the mutagenic activity of NaOCl in *Cyprinus carpio* with the Comet test and MN test.

NaOCl is highly toxic to *A. microlepis* and the average LC_{50} of NaOCl for *A. microlepis* was calculated as 0.6343 mg/L (Aksu et al. 2008). According to this study, there is an increase in micronucleus and double nucleus formation together with increasing dose. Guzzella et al. (2004) determined the in vitro mutagenic effect of three disinfectants including NaOCl using the salmonella mutagenicity test, microtox and mutatox methods. Bolognesi et al. (2004) reported that NaOCl increases the MN frequency in *Dreissena polymorfa*.

In contrast to the above-mentioned studies, Crebelli et al. (2005) stated that NaOCl does not show mutagenic activity in *Allium cepa* (using the anaphase aberration test) or in *Tradescantia* (using the micronucleus test).

In conclusion, our data provide evidence that NaOCl increases the chromosomal aberration rate,

Table 2 The micronucleus frequency, nuclear division and nuclear cytotoxic division index in human lymphocytes treated with NaOCl

Test substance	Treatment		Binucleated cells scored	Distribution of cells according to number of nuclei				Distribution of BN cells according to the no. of MN				MN/cell (%) ± SEM	NDI	Apoptotic cells	Necrotic cells	NCDI
	Period (h)	Dose (μg/mL)		1	2	3	4	1	2	3	4					
Negative control	–	–	2,000	606	1,013	114	247	1	0	0	0	0.05 ± 0.01	1.98 ± 0.34	0	0	1.98 ± 0.25
PC (MMC)	48	0.5	2,000	1,418	528	26	28	170	19	1	0	10.55 ± 1.27	1.33 ± 0.90	23	27	1.35 ± 1.10
NaOCl	48	0.5	2,000	755	868	257	122	12	1	0	0	0.70 ± 0.14*	1.87 ± 0.34	24	26	1.90 ± 0.45
	48	1	2,000	1,045	710	189	56	22	4	0	0	1.50 ± 0.25*	1.62 ± 0.48	31	29	1.65 ± 0.41
	48	1.5	2,000	970	941	208	81	26	2	0	0	1.50 ± 0.31*	1.70 ± 0.68	27	25	1.72 ± 0.76
	48	2	2,000	1,027	716	195	62	32	6	0	0	2.20 ± 0.37*	1.64 ± 0.55	42	54	1.69 ± 0.41
	48	2.5	2,000	826	906	188	80	38	9	1	1	3.15 ± 0.40*	1.76 ± 0.45	44	43	1.80 ± 0.53

NDI nuclear division index, *NDCI* nuclear cytotoxic division index, *SEM* standard error of the mean

* Significantly different from the negative control $P < 0.05$ (Dunnett's *t*-test)

Fig. 5 Photomicrographs of (**a**) binucleate cell with one micronucleus; **b** apoptotic cell with nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane; **c** necrotic cell with damaged/irregular nuclear membrane

MN formation and cytotoxicity in human lymphocyte cells in vitro at concentrations approximately 33 times lower than that found in drinking water.

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