HIGH GERMICIDAL AND LOW MUTAGENIC EFFECT OF SODIUM HYPOCHLORITE

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INTRODUCTION

Sodium hypochlorite was first registered for commercial use as an antimicrobial pesticide in 1957(U.S.E.P.A., 1991). The aqueous solution of Sodium hypochlorite with 5.25% in concentration is commonly used as a house-hold bleaching agent. Sodium hypochlorite is registered with the Environmental Protection Agency for use as a sanitizing agent, disinfecting agent. The chemical has many usages in the area of food processing, laundry services, agricultural settings, gardens, animal facilities, hospitals, and human drinking water supplies (Weber, 1997). Household bleaches usually contain 3% to 6% sodium hypochlorite whereas industrial and institutional bleach applications are typically 10%~12% active (Fletcher, 2001). The mechanism of disinfection has not been completely understood, but researchers have proposed enzyme disruption, protein denaturation, and nucleic acid inactivation for such germicidal activity (Dychdala, 1991). Bacteria, molds, and mildews are inhibited and killed by sodium hypochlorite (Herndon, 1991). The use of bleach in prevention of transmissible diseases in health care have been proven to be very effective (Spire, 1984). The acquired immunodeficiency syndrome virus - III/ lymphadenopathy- associated virus, was inactivated by the application of this bleaching agent (McDougal et al, 1985). Sodium hypochlorite was also effective for sanitation of many other viral diseaases including african swine fever virus, equine viral arteritis virus, porcine reproductive and respiratory syndrom virus, and the african horse sickness virus (Shirai, 2000). Hypochlorite appears to have evolved from oxidative intermediates in the lysosome of eucaryotes to function not only for phagocytosis,but also for disinfection. Hypochlorite is produced in lysosome from hydrogen peroxide and chloride by the enzymatic activity of myeloperoxidase at the time of respiratory burst and at the time of biological crisis for disinfection and

phagocytosis. In multicellular system particularly in animal, further coordination of phagocytosis mechanism in the cell with general endocrine system for adjustment of metabolic rate with the digestive activity of the organ and phagocytosis activity of the cell. Thyroid hormone secretion was increased in such occasion. by enhancement of peroxidase in the follicular cell of thyroid gland for formation of hypoiodite from iodide. Subsequently, iodination of tyrosol was achieved by thyroxin at the time of endocrine alert. Because of the increased importance of sodium hypochlorite as disinfectant and cleaner in home and environment, the biosafety of this chemical compound particular genotoxic effect of it is of great concern to many users. Current study intends to reevaluate both germicidal effect and genotoxic effect of this chemical to the test strain TA 98. In addition the strandbreaksing effect of this chemical to Simian virus 40 viral DNA.will be examined.

MATERIALS AND METHODS

Bacterial Strain and DNA preparation

Salmonella Mutagenesis Test Strains TA98 was obtained from Dr Ames Laboratory, University of California at Berkeley. SV-40 viral DNA was purchased from the Bethesda Research Laboratory, Life Technologies Inc. (BRL) . The viral DNA was mostly in the Form-I (more than 95%) and partly in the Form-II (less than 5%) at the time of purchase. Electrophoresis purity grade Agarose powder was obtained from the Bio-Rad Laboratory.

Salmonella Bacterial Mutagenecity Assay

Mutagenic assay was performed according to the method of Ames et al (1975) . A *Salmonella* mutant test strain, TA98 was streak cultured in MG agar plate (1,5% agar, 2% dextrose, 0.00978% $MgSO_4$, 0.183% citric acid,1% K_2P0_4, and 0.229% $NaHNH_4PO_4$) with the addition of histidine (260 µM), biotin (3 µM) and ampicillin (25 µg/ml) for maintenance. For preparation of frozen stocks, liquid culture of either TA98 was made by shaking culture of a single colony of tester strain to early stationary phase in 40 ml of the Oxoid Nutrient Broth #2. The 40 ml culture was mixed with 7 ml glycerol and dispensed 1 ml per microtube for storage at -80^0C in a deep freezer. For the mutagenecity test, one ml of frozen stocks was diluted into 80 ml of Nutrient broth #2 for culture in a shaker incubator. Following 5 to 6 hours of

incubation at 30^{0}C and 200 rpm in a shaker incubator, mid-logarithmic phase of the growth was obtained. Preincubation was performed by mixing of the 0.1 ml of the cultured bacterial strain with sequentially diluted sodium hypochlorite from the concentration of 0.00078125% to 0.4% in test tubes (13 x 100 mm) for 30 minutes at room temperature in a shaker. Thereafter, 0.5 ml of salts mixture containing 0.1 mM phosphate buffer (pH 7.4 or pH 3.4), 33 mM potassium chloride and 8 mM magnesium chloride was mixed with the preincubated mixture in each tubes. After the final addition of hot top agar (55° C) containing 0.5% saline solution, 0.6% agar, 0.5 mM histidine and 0.5 mM biotin for vigorous vortex for 30 seconds, the mixture was coated on the top of MG plates for incubation at 37° C. Number of colonies formed by reverse mutation were recorded after 48 hours and 72 hours of incubation.

DNA Strandbreak Assay

DNA strandbreak assay was performed by electrophoresis for separation of SV-40 viral DNA into 3 forms: Form-1, Form-2 and Form-3 for quantitative analysis under a densitometer (Alder et al., 1973) For electrophoresis, a TBE buffer containing 1.08% Tris-hydroxide-methyl-aminomethane (Tris-base), 0.55% Boric acid, and 0.0761% Ethylenediamine tetraacetic acid (EDTA) was prepared with pH adjusted 7.5. A 0.8% agarose gel for electrophoresis was made by mixing 0.9 g of agarose in 115 ml TBE buffer and heated to boiling point for complete dissolution of agarose in the solution. After the prepared agarose solution was cooled down to 60^{0}C, it~was poured into the mold (15 x 15 cm glass plate framed and sealed by tape) with a 20 well comb (teeth size for each well; 1 mm x 3 mm x4 mm) hanging 1 mm above it. The prepared agarose plate was kept in a refrigerator until the time of use. For incubation of DNA with test chemicals, TBNE buffer containing 1.24% Tris-base, 0.2922% sodium chloride, and 0.38% EDTA was prepared. The pH of buffer was usually adjusted to 7.5 with 10 N NaOH. Fifty ml of the TBNE buffer was used to dissolve o.4 g of hydroquinone or ascorbic acid. Thereafter the solution was diluted into the concentration of 0.2%, 0.1%, 0.05%, 0.025%, 0.0125% by sequential dilution of the original hydroquinone solution or ascorbic acid solution. Ten µl of each sequentially diluted test

samples were transferred into microcentrifuge tubes. After the addition of 10 µl DNA solution containing 1.7204 µg of DNA in various concentration of hydroquinone, the mixture was gently mixed by tapping and incubated for 8 or 24 hours in a refrigerator. Thereafter, 10 µl of loading medium containing 0.025% BPB, 0.025% Xylene cyanol, and 2.5% Ficol "Type 400" was added and mixed again. Finally, 15 µl of the final mixtures (sample mixed with loading medium) were gently and quietly added to the bottom of wells in the agarose gel which was placed in a electrophoresis apparatus under TBE buffer solution. After electrophoresis for 4 to 6 hours at constant voltage of 80 DCV (40 mA), the gel was stained in Ethidiurn bromide solution (0.5 µg/ml water) for 4 hours or overnight in a tank. Following destaining with 1 mM magnesium sulfate for 20 to 30 minutes, the gel was placed on a ultraviolet transilluminator for fluorescent photography with the wave length of 300 nm. A Polaroid camera with Kodak #22 Wratten filter and a sensitive Polaroid film, type 55 P/N black and white film was used for photography. A LKB Gelscan XL laser beam densitometer was used for scaning, integration and data management of the fluorescent photography.

RESULTS

Salmonella **Bacterial Mutagenecity Assay**

In the bacterial mutagenicity test, significant reduction in the number of reverse mutants suggesting lethal effect of hydrochlorite to reverse mutants were observed at most of the test groups (6 out of 8) in the concentration level between 0.003125% to 0.40% (Group 4, 5, 7, 9, 10, 11). Particular the germicidal effect of the chemical was 100% effective at the concentration level higher tham 0.1% (Groups 9, 10, 11). On the contrary, a significant increase of reverse mutants observed at the concentration level of 0.05% suggesting a significant mutagenecity effect at that concentration (Group 8).

DNA Strandbreak Assay

Two DNA bands identified as form-1 DNA (closed circular, superhelix DNA) and form-2 DNA (nicked circular DNA) in the ratio of about 93% and 7 % were normally obtained following electrophoresis of SV4O viral DNA isolated from the host kidney cell culture of monkey infected with the virus. Incubation of this DNA probe with various concentrations of hypocholite resulted in the

strandbreaking effect to the DNA probe resulting in increase of Form II DNA by single strandbreaks and increase of Form III DNA by the double strandbreaks which could be assayed by electrophoresis followed by fluorescent photography and densitometry in Table 1. When the concentration of sodium hypochlorite for incubation with the DNA probe was increased at the lower levels between 0.003125% to 0,00625 % the proportion of Form-I DNA reduced from 92.9% to 67.7% and the proportion of Form-II DNA increased from 7.1% to 32.3%, suggesting the increase of single strand break by the minor destruction of the DNA probe. At higher concentration levels from the 0.0125% to 0.10% of chemicals, the new DNA band, Form-III (linear form) appeared and gradually increased its proportions from 10.7 to 48.3% of total DNA, suggesting the increase of double strandbreaks by the serious destruction of the DNA molecule. (Table 1).

DISCUSSION

Oxygen and radicals are the most important class of mutagens contributing to aging and cancer (Ames 1982). Hypochlorite which has been converted from hydrogen peroxide and chloride ion in the lysosome by myeloperoxidase have similar DNA strandbreaking effect as the hydrogen peroxide (Yang et al. 1990), however the mutagenicity effect of the chemical appears to have reduced following such chemical change by substitution of hydroxyl group with the chlorine element in the molecule. It is possible that the DNA damage induced by this chemical did not result in the error of repair to cause mutagenecity. In addition, the germicidal effect might have been enhanced by binding of hypocholite ion to enzyme systems in cell to cause lethal effect Significant effect to reduce reverse mutants were seen at considerably wide concentration levels between 0.0015625% and 0.40% (Groups 3 to 11). The germicidal effect of the chemical was particularly remarkable at the concentration levels higher than 0.1% (approximately 1:50 dilution of 5.5% Clorox), where the 100% lethal effects of the sodium hypochlorite were obtasined (Groups 9, 10, 11). Nevertheless, a significant increase of colony formation (reverse mutants) was also seen in one particular concentration of 0.05% (Group 8) which indicate a mutagenic effect at this very limited concentration level. Our result obtained in the *Salmonella* bacterial mutagenecity test demonstrated that the major effect of sodium hypochlorite to the *salmonella* TA98 is germicidal but not mutagenetic. Early studies cited by the Occupational Safety

and Health Administration (OSHA) which recommended the use the Clorox for disinfection at the dilution of the 1:10 in the circumstances of blood or body fluid spills (Eden, 1997). The recommended concentrations of household bleach (5.25%) for each species of bacteria are 1:500 (bacteria), 1:100 (Bacillus subtilis species), 1:50 (Mycobacterium), 1:20 (virucidal), 1:10 (Mycobacterium tuberculocide), and 1:1 (high level disinfectant for bacterial spores) respectively (Rutala, 1996). The chemical was also effective for the inactivation of Human Immunodeficiency Virus (HIV),at the concentration level between 1:100(500ppm) and 1:10(5000ppm) of household bleach(CDC,1987). This concentration is also equivalent to the concentration level of sodium hypochlorite in the current study to induce 50% single strand break in DNA. Although the the mutagenecity effect of the chemical was limited to one particular concentration level of the chemical, however the degree of significance is very high to suggest that it has to be cautious in the application of this chemical for disinfection. Earlier studies on the mutagenecity effect of this chemical had some contradictory results between them. Bacterial mutagenecity test of the sodium hypochlorite by some investigators demonstrated warning positive results (Ishidate et al., 1994, Wlodkowski and Rosenkranz 1975). Nevertheless, negative results for the mutagenecity were obtained by testing of the chemical with TA97 and TA 102 test strains also (Fujita and Sasaki 87, Le Curieux, Marzin, and Erb, 1993), When TA98 and TA100 were used for mutagenecity test of raw water and drinking water samples from 5 treatment plants using lake waters treated with sodium hypochlorite in northern Italy, there are increasing mutagenecity in winter water samples (Monarca, et al. 1998).

CONCLUSION

Sodium hypochlorite (Clorox) has demonstrated its strong germicidal effect to the *Salmonella tester* tester strain TA98 in the current study. The germicidal effects of the chemical to the *Salmonella* TA98 are in the concentration level higher than 0.003128 with 100% lethal effect which can be achieved at the concentration higher than 0.1%. The chemical has shown a strong strandbreaking effect to the *Simian* virus 40 viral DNA also. Nevertheless, the mutagenic effect of the chemical was also noted in one limited concentration (0.05%) which is one step close to the 100% lethal effect. For this reason, it has to be cautious in the application of sodium hypochrolite for disinfection.

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Table 1. Germicidal and Mutagenecity Effect of Sodium Hypochlorite in TA98

Group	Hypochlorite Concentration		Numbers of Revertants	
			Observed at 48 hours	Observed at 72 hours
No.	mM	(%)	Mean ± S.D. (est. t #)	Mean ± S.D. (est. t #)
1	0.00000	(0.00000000)	34.5 ± 2.6 (Neg. Cont.)	57.8 ± 6.9 (Neg. Cont.)
2	1.03125	(0.00078125)	36.0 ± 6.3 (t= +0.44)	51.0 ± 5.0 (t= -1.60)
3	2.0625	(0.0015625)	37.0 ± 3.3 (t= +1.19)	56.3 ± 3.8 (t= -0.38)
4	4.125	(0.003125)	19.8 ± 5.3 (t= -4.98)	39.3 ± 3.5 (t= -4.78)
5	8.25	(0.00625)	24.3 ± 2.2 (t= -5.99)	43.5 ± 3.5 (t= -3.70)
6	16.5	(0.0125)	33.8 ± 4.4 (t= -0.27)	53.8 ± 3.6 (t= -1.03)
7	33.0	(0.025)	14.5 ± 6.9 (t= -5.43)	24.3 ± 7.5 (t= -6.57)
8	66.0	(0.05)	98.0 ± 30.0 (t= +4.27)	127.0 ±33.3 (t= +4.07)
9	132.0	(0.10)	0.0 ± 0.0 (t=-26.5)	1.5 ± 1.3 (t=-16.04)
10	265.0	(0.20)	0.0 ± 0.0 (t=-26.5)	0.0 ± 0.0 (t=-26.5)
11	530.0	(0.40)	0.0 ± 0.0 (t=-26.5)	0.0 ± 0.0 (t=-26.5)
12	Daunomycin 0.05μg/100ml		132.3 ±105.7 (Pos. Cont)	336.0±102.5 (Pos. Cont.)

Note: # : estimated $t = [(Mean_1 - Mean_2) - 0] / [Sp^2 (1/n_1 + 1/n_2)]^{1/2}$

$Sp^2 = [(n_1-1)S_1^2 + (n_2-1)]S_2^2] / [n_1 + n_2-2)$

Est. t value is significant when $t > +1.984$ or $t < -1.984$

at $df = n_1 + n_2-2 = 6$ and $\alpha = 0.95$

Table 2. DNA Strndbreaks induced by incubation of SV40 viral DNA (0.011 uM) with Sodium Hypochlorite. (Incubation for 8 hours at 4°C and pH 7.5; Electrophoresis with 100 DCV and 50 mA for 8 hours).

Sample No.	Hypochlorite Concentration %	µM	Proportion of DNA (%) Form I	Form III	Form II
1	0.00000000	0.0	92.9	0.0	7.1
4	0.003125	284	88.2	0.0	11.8
5	0.00625	568	67.7	0.0	32.3
6	0.0125	1135	44.3	10.7	21.6
7	0.025	2270	35.0	17.6	24.4
8	0.05	4540	49.2	18.1	33.3
9	0.10	9080	16.6	48.3	35.1

Effective dose to cause 25% strand break on Form I DNA is estimated to be 565.2 µm ($SBED_{-25\%}$) and that to cause 50% strand break on Form I DNA is estimated to be 5417.4 µm from this experiment.

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