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Genotoxicity Testing of Two Anticaking Agents: Sodium and Potassium Ferrocyanide *in vitro*

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ABSTRACT Anticaking agents are mainly used in food to prevent agglomeration in certain solids, permitting a free-flowing condition. Sodium and potassium ferrocyanide are among the popular anticaking agents which are used in table salt. The present study focuses on the genotoxic effect of sodium and potassium ferrocyanide on human lymphocyte. Human lymphocytes were exposed to the sodium and potassium ferrocyanide at concentrations ranging from 0 to 10 mM for 3 h at 37ºC. Cytotoxicity was evaluated by trypan blue dye exclusion test and resazurin test was carried for cell viability. To assess the extent of DNA damage comet assay was performed. The results show that potassium ferrocyanide was cytotoxic and genotoxic at the concentrations tested, whereas, sodium ferrocyanide was non genotoxic.

1. INTRODUCTION

Anticaking agents consist of substances that are added to fine-particle solids, such as food products like table salt, flours, coffee, and sugar to prevent them from agglomeration in certain solids, permitting a free-flowing condition (Yeong-Lin et al. 1993). They rarely contain nutritional value and only a small proportion of these additives find their way into the food used for the purpose.

Safety evaluation of certain food additives and contaminants prepared by Joint FAO/ WHO Expert Committee on Food Additives World Health Organization, Geneva 2011 (FAO/ WHO 2011) has envisaged the need for genotoxicity and mutagenicity tests. These tests are effective genotoxic endpoints for interpreting and correlating to human. Literature on toxicity studies of different anticaking agents is scarce and often inconclusive. Animal inhalation studies have shown that silica, an anticaking agent, creates potential health hazards like COPD (Compulsive obstructive pulmonary disease), reversible inflammation, granuloma formation and emphysema but do not cause progressive fibrosis of the lungs (Merget et al. 2002). Experiments on rat have come up with no such toxicity data up to 5000 mg/kg body weight (ECETOC 2006). Water soluble iron cyanide compounds are widely in use as anticaking agents especially in feedstuffs (FAO/ WHO 1975). Sodium ferrocyanide (E 535) and potassium ferrocyanide (E 536) are complex cyanides that belong to the ferrocyanide group of additives. They are in use for years as anticaking agents in salts for human consumption. They are approved by JECFA/ WHO 1975 (FAO/ WHO 1975) and evaluated by SCF 1990 (SCF 1990) with an ADI of 0-0.025 mg/kg body weight. According to Food Adulteration Act (PFA), India 1955, calcium, sodium and potassium ferrocyanide are permitted in salt and its substitutes with maximum amount fixed at 20 mg/kg of food.

Genotoxicity testing of these two anti caking agents (sodium and potassium ferrocyanide) utilizing Ames test, and an *in vitro* cytogenetic assay with human lymphocytes and a mouse lymphoma L5178Y cell assay was found to be negative (EC 2001). A carcinogenicity study in rats with doses up to 5000 ppm (0.5 %) and a teratogenicity study did not indicate carcinogenic or teratogenic potential as well (EC 2001). A majority of the reports published earlier were not according to GLP (Good Laboratory Practice). Therefore, we deemed important to study the genotoxicity of these two anticaking agent on human lymphocyte cells. Since the alkaline comet assay introduced by Singh et al. (1988) is a rapid, simple and sensitive procedure for quantitating DNA lesions in mammalian cells, we have evaluated the DNA damaging poten-

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tial (if any) of sodium and potassium ferrocyanide using comet assay. In addition, cytotoxicity was also studied.

2. MATERIAL AND METHODS

2.1. Chemicals

Sodium ferrocyanide (CAS no 13601-19-9) and potassium ferrocyanide (CAS no 13943-58-3) were procured from Hi Media Ltd., Mumbai, India. RPMI- 1640 medium was procured from Invitrogen Corporation, USA. Resazurin, Ethidium bromide, histopaque, low melting point agarose (LMPA), methyl methane sulphonate and trypan blue were purchased from Sigma-Aldrich Co. (USA). Dimethyl sulfoxide (DMSO), disodium EDTA, sodium chloride (NaCl), sodium hydroxide (NaOH), triton X-100, trizma base and hydrochloric acid (HCl) were purchased from Merck India and were of analytical grade.

2.2. Test System

Human peripheral blood was obtained by venipuncture from a healthy donor into vacutainers. Lymphocytes, isolated from fresh peripheral blood samples, were used for the experiment.

2.3. Isolation of Mononuclear Cells From Blood

Peripheral blood have been taken from healthy male human volunteers (non-smokers and not under any medication), average age 23±1 years, on each day of analysis into vacutainers (heparin). Lymphocytes were isolated from the blood according to the method described by Boyum (1976), with slight modifications (Ghosh et al. 2010). Blood (2 mL) was diluted with equal volume of PBS and 2 mL of the diluted blood was layered gently over 2 mL of Histopaque and kept aside for 25 min. The tube was centrifuged at 1000 rpm for 40 min. The buffy coat was aspirated into 4 mL of PBS and was centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the lymphocyte pellet was suspended at a concentration of 10^6 cells/ mL in RPMI-1640 medium. Ethical clearance was obtained from the institutional ethics clearance committee of the University of Calcutta.

2.4. Treatment of Cells

Stock solutions of sodium ferrocyanide and potassium ferrocyanide were prepared by dissolving in RPMI-1640 media. The human lymphocytes ($5x10^5$ cells/ mL) were exposed to three concentrations (1, 5 and 10 mM) of the test chemicals for 3 h at 37º C. Positive (50 µM MMS) and negative (only RPMI medium) controls were simultaneously maintained. After incubation, the lymphocytes were harvested by centrifuging at 2000 rpm for 10 min and the cells were suspended in PBS. Two aliquots were prepared, one used for the comet assay and the other was used for the viability and cytotoxicity tests.

2.5. Cell Viability Assays

2.5.1. Trypan Blue Dye Exclusion Test

Viability of the cells were evaluated using trypan blue dye exclusion method (Tennant 1964). 15µL cell suspension was mixed with same volume of trypan blue (0.4%) dye, kept for 5 minutes and the percentage of viable cells were scored under microscope using haemocytometer.

2.5.2. Resazurin Cell Viability Assay

The cytotoxicity of the chemicals was determined by using the resazurin cell viability assay kit according to the supplier's guideline. Briefly, $5x10^5$ cells were seeded in a volume of 100 µl into wells of 96 well ELISA plate (Round bottom). 10µL resazurin solution was added to each well, mixed gently by tapping and was incubated at 37°C for another 2 hours. The plate was read at 595nm wavelength and at a reference wavelength of 655 nm in an ELISA plate reader (BIO-RAD, USA iMark™ Microplate Absorbance Reader). The results were converted to percentage viability of cells and compared to the untreated control.

2.6. Analysis of DNA Damage Using Comet Assay (Single Cell Gel Electrophoresis)

The extent of DNA damage was studied by the comet assay or Single Cell Gel Electrophoresis (SCGE) according to the method of Singh et al. (1988) with modifications (Ghosh et al.

2010). The slides were stained with 50–75 μL of ethidium bromide (20 μg/ mL) for 5 min and rinsed in ice-cold water to wash off excess stain. Slides were scored using image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1). The microscope was connected to a computer through a charge-coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. Images of 150 (50x 3) cells per concentration (n=3 for each concentration) were analyzed. The median values of tail DNA (%) and tail extent (μm) were scored from each slide and expressed in terms of means ± SEM for each treatment group (Kumaravel and Jha 2006).

2.7. Statistical Analyses

For all statistical analysis, Sigma Stats. 3 software (SPSS Inc., Chicago, Illinois, USA) was used and the level of significance was established at 0.05. Analysis of Variance using the median value of each slide at each concentration level was done using one-way ANOVA to examine the differences in the overall concentrations with the negative control. This would detect the minimum effective concentration of the chemicals to induce DNA damage.

3. RESULTS

The trypan dye exclusion method for cell viability test did not show any significant cytotoxicity in human lymphocytes treated with different concentrations of sodium and potassium ferrocyanide (1, 5 and 10mM). The percent cell viability varied between 97– 99% in the sodium/ potassium ferrocyanide exposed lymphocytes (Figs. 1, 2). Sodium and potassium ferrocyanide salts responded differentially in the resazurin cell viability assay (Figs. 1, 2). The value for potassium ferrocyanide ranged between 77 to 81 % and was significantly low ($p \leq 0.05$) at all concentrations. For sodium ferrocyanide cell viability was significantly low ($p \leq 0.05$) at the highest concentration only.

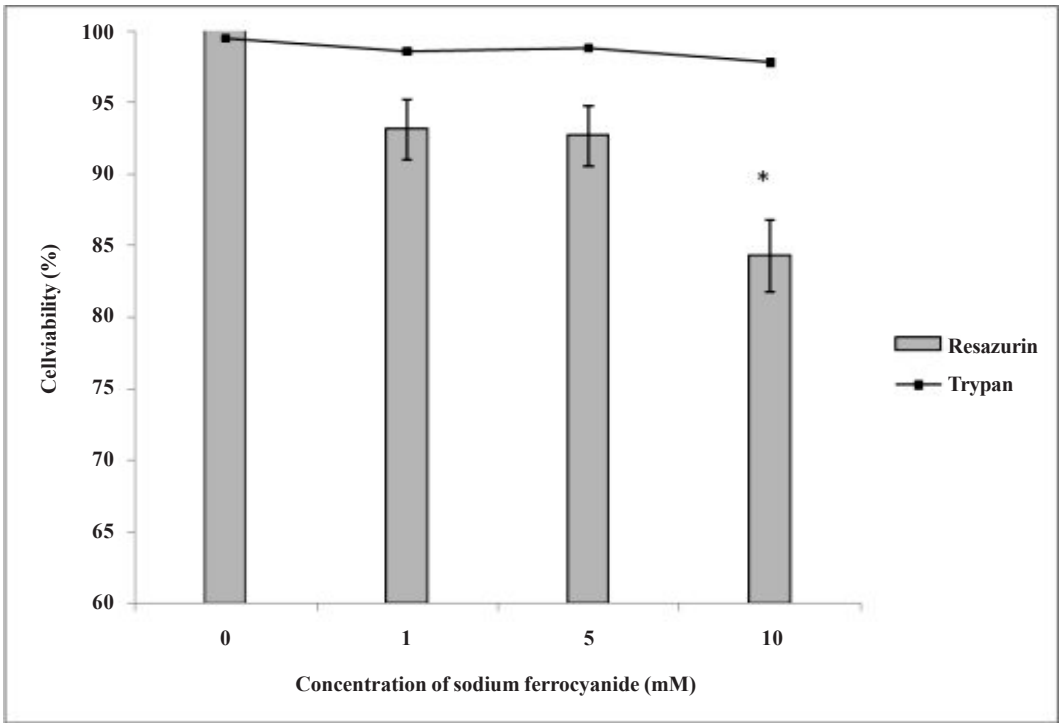

Fig. 1. Trypan blue dye exclusion test and resazurin cell viability assay, cytotoxicity induced in human lymphocytes treated with sodium ferrocyanide; $^{*}p \leq 0.05$

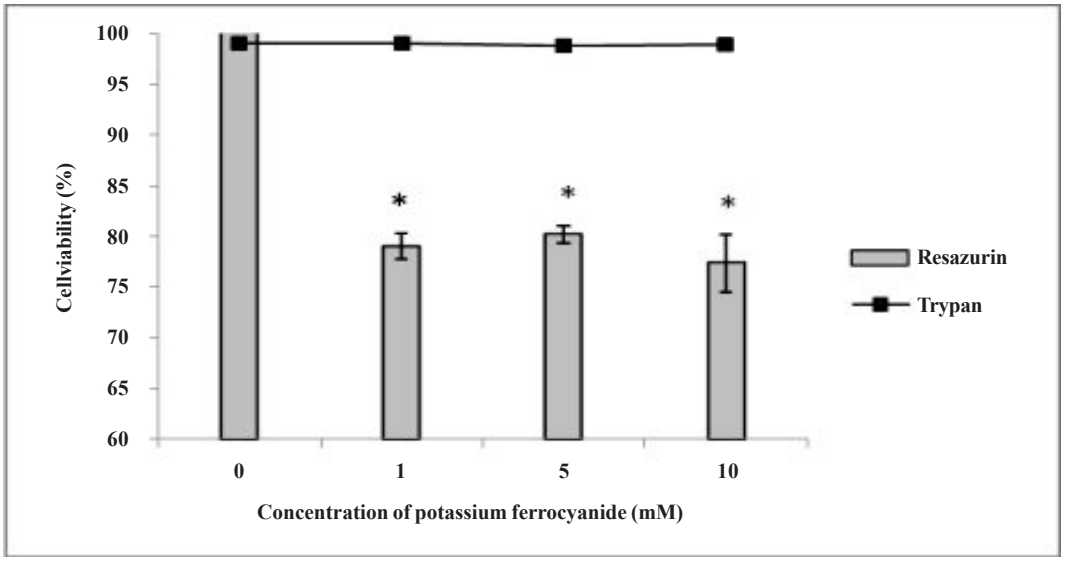

Fig. 2. Trypan blue dye exclusion test and resazurin cell viability assay, cytotoxicity induced in human lymphocytes treated with potassium ferrocyanide; $^{*}p \leq 0.05$

The effect of the sodium and potassium ferrocyanide on comet parameters is presented in Tables 1 and 2 respectively. Potassium ferrocyanide produced a concentration-dependent increase in DNA damage, as measured by the % tail DNA (Table 1) and tail extent (Table 2). The values were statistically significant at concentrations 5 mM and above. The values of the comet parameters of sodium ferrocyanide treated cells were not significant (Tables 1 and 2) when compared to the negative control values. The positive control (50 μM of MMS) showed a higher increase in DNA damage.

4. DISCUSSION

Sodium and potassium ferrocyanide are anti caking agents authorized for human consumption by European Council Directive 95/2/EC (OJ 1995) as well as by the Prevention of Food Adulteration (PFA) Act 1954, modified in 2011 of India. They are allowed to be used in salt, salt substitutes and also in seasoning and condiments (Codex Alimentarius Commission 2011) with maximum permissible level of 20 mg/kg. There are a few reports on the genotoxicity of these two anticaking agents. In the present study, the results show that potassium ferrocyanide is cytotoxic and genotoxic at higher concentrations, while sodium ferrocyanide is found to be non-genotoxic at all the concentrations tested. Long-term *in vivo* studies are on way to assess the genotoxicity of these anti caking agents.

Table 1: Effect of treatment of sodium and potassium ferrocyanide on % tail DNA of human lymphocyte cells as assessed in comet assay

Concentration (mM)	*Tail DNA (%)*[a]	
	Sodium ferrocyanide	*Potassium ferrocyanide*
0	1.66 ± 0.4	2.16 ± 0.15
1	2.08 ± 0.31	3.42 ± 0.35
5	2.34 ± 0.37	3.55 ± 0.12*
10	2.49 ± 0.08	4.22 ± 0.5*

[a] Values are average of median of 150 cells (50 x 3) ± SEM
*Significant difference; $p \leq 0.05$ when compared to control (Analysis of Variance Test), value for MMS (50μM) = 35.79 ± 1.92

Table 2: Variation of tail extent with concentrations of sodium and potassium ferrocyanide

Concentration (mM)	*Tail Extent (μm)*[a]	
	Sodium ferrocyanide	*Potassium ferrocyanide*
0	0.02 ± 0.00	0.01 ± 0.00
1	0.04 ± 0.01	0.03 ± 0.00*
5	0.06 ± 0.00	0.05 ± 0.01*
10	0.05 ± 0.02	0.04 ± 0.01*

[a] Values are average of median of 150 cells (50 x 3) ± SEM
Significant difference; $p \leq 0.05$ when compared to control (Analysis of Variance Test), value for MMS (50μM) = 5.08 ± 0.56

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