

## AMELIORATION BY BLACK TEA EXTRACT OF SODIUM FLUORIDE INDUCED HEMOLYSIS OF HUMAN RED BLOOD CELL CORPUSCLES

RJ Verma,<sup>a</sup> MH Trivedi, NJ Chinoy<sup>†</sup>

Ahmedabad, India

**SUMMARY:** The ameliorative effect of black tea extract on sodium fluoride (NaF) induced hemolysis was studied *in vitro*. Venous blood samples from twenty healthy, 25–30-year-old, well-nourished male donors residing in a nonfluorotic area of Ahmedabad, India, were collected in EDTA vials for the preparation of red blood cell (RBC) suspensions in normal saline. When 2.0 mL of RBC suspensions were treated for 4 hr at 37°C with 0.05–0.5 mL of 4 mg NaF/mL in normal saline (50–500 µg NaF/mL in a final normal saline volume of 4.0 mL), they exhibited a significant dose-dependent increase in hemolysis. Addition of black tea extract (100 µg/mL in final 4.0 mL volume) to the RBC suspensions did not significantly affect hemolysis. However, concurrent addition of 0.5 mL of NaF (500 µg/mL in final 4.0-mL volume) and 0.025–1.0 mL of black tea extract (2.5–100 µg/mL in final volume) caused a significant reduction in the percent hemolysis with maximum amelioration occurring at 40 µg extract/mL. The higher concentrations of tea extract (50–100 µg/mL) did not produce further amelioration, but the percent hemolysis remained significantly lower than that of the NaF treated RBC suspensions.

Keywords: Amelioration of hemolysis; Black tea extract; Cytotoxicity; Hemolysis; Human red blood cell (RBC) corpuscles; Sodium fluoride hemolysis.

### INTRODUCTION

Toxic effects of excessive ingestion of fluoride (F) are matters of grave international concern since many countries have regions of endemic fluorosis.<sup>1</sup> In India 17 of 32 states are reported to have endemic fluorosis. In addition to its effects on bones and teeth, fluoride also causes serious disturbances in a number of organs, including liver, kidney, and reproductive organs, and can cause oxidative damage through formation of free radicals.<sup>2</sup>

In a rapidly developing country like India, tea is one of the most common and widely consumed beverages. It contains large numbers of antioxidant polyphenols; typically 93% of total tea phenolic compounds are flavonoids.<sup>3–4</sup> Earlier investigation has revealed that one or two cups of tea have the same ‘radical scavenging capacity’ as five portions of fruits and vegetables or 400 mg of vitamin C equivalents.<sup>5–6</sup> Therefore, we undertook the present investigation to determine the ameliorative effects of black tea extract on sodium fluoride (NaF) induced cytotoxicity of red blood corpuscles (RBC) *in vitro*.

### MATERIALS AND METHODS

**Black tea extract:** Black tea (Brook Bond yellow label) purchased from the local market was extracted as per WHO protocol CG-06.<sup>7</sup> About 15 g of the tea was powdered and suspended in 100 mL of deionised water heated to 90°C for 30 min. After cooling, the mixture was filtered twice through Whatman No. 1 filter paper. The filtrate was collected and evaporated to dryness in an air oven at 90°C and

<sup>a</sup>For Correspondence: RJ Verma, Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad-380009, Gujarat, India. E-mail: zooldeptgu@satyam.net.in.

<sup>†</sup>Deceased 8 May 2006.

used for the preparation of black tea extract.<sup>8</sup> A 1% solution of the black tea extract used in the experiment contained 0.58 ppm of fluoride.

*RBC suspension:* Blood samples were obtained with voluntary consent from 20 well-nourished, healthy adult male donors, age 25–30 years, residing in nonfluorotic areas of Ahmedabad and not having any sign of dental fluorosis. Venous blood was collected in vials containing ethylenediaminetetraacetic acid (EDTA), diluted with normal saline (0.9% NaCl) and centrifuged at 300 g for 10 min. The RBC pellets were washed twice and finally suspended in normal saline to have a cell density of  $2 \times 10^4$  cells/mL.

*NaF solution:* For the experiments, solutions of 4 mg NaF/mL were prepared in normal saline.

*Black tea extract:* Also for the experiments, solutions of 0.4 mg/mL of black tea extract were prepared in normal saline.

*Methods:* To determine the effect of NaF on RBC, the following sets of spectrophotometric tubes were prepared:

- 1 *Control tubes:* These tubes contained 2.0 mL RBC suspension and 2.0 mL normal saline.
- 2 *NaF treated tubes:* Different volumes of the NaF solution (0.05–0.5 mL) were mixed with 2.0 mL RBC suspensions, and the final volume was made to 4.0 mL with normal saline. The concentrations of NaF in the 4-mL final volume therefore ranged from 50 to 500  $\mu\text{g/mL}$ .

To determine the effect of black tea extract on NaF induced cytotoxicity in RBC, following sets of spectrophotometric tubes were prepared:

- 1 *Control tubes:* These tubes again contained 2.0 mL RBC suspension and 2.0 mL normal saline.
- 2 *NaF treated tubes:* 0.5 mL of the NaF solution was mixed with 2.0 mL RBC suspension. The final volume was made to 4.0 mL with normal saline. Therefore the concentration of NaF in the final volume was 500  $\mu\text{g/mL}$ .
- 3 *Black tea extract treated tubes:* 1.0 mL black tea extract was mixed with 2.0 mL RBC suspension. Final volume was made to 4.0 mL with normal saline. Therefore the concentration of black tea extract in the final volume was 100  $\mu\text{g/mL}$ .
- 4 *NaF and black tea extract treated tubes:* 0.5 mL of NaF solution and 0.025–1.0 mL black tea extract were mixed with 2.0 mL RBC suspension. The final volume was made to 4.0 mL with normal saline. Therefore the concentrations of NaF and black tea extract in the final volume were 500  $\mu\text{g/mL}$  and 2.5–100  $\mu\text{g/mL}$ , respectively.

All tubes were incubated at 37°C for 4 hr with intermittent shaking. Morphological alterations in RBC were observed microscopically by staining RBC smear with Leishman's stain.

Absorbance of the supernatants obtained after centrifuging the incubated tubes at 300 g for 10 min, were read spectrophotometrically at 540 nm.<sup>9</sup> Percent hemolysis was calculated by the formula below.<sup>10</sup>

$$\text{Percent hemolysis} = \frac{\text{Absorbance of individual tubes}}{\text{Absorbance with 100\% hemolysis}} \times 100$$

To achieve 100% hemolysis, 2.0 mL distilled water was added to 2.0 mL RBC suspension.<sup>11</sup> Percent reduction in hemolysis with different concentrations of black tea extract was calculated using the following formula.<sup>11</sup>

$$\text{Percent reduction in hemolysis} = \frac{A-B}{A} \times 100$$

A = sodium fluoride induced hemolysis;  
B = hemolysis caused by concurrent addition of sodium fluoride and black tea extract.

Statistical analysis of the data was performed using Student's t test. Values of  $p < 0.05$  were considered statistically significant.

## RESULTS

In control tubes RBC appeared as spheres or biconcave discs. The cells remained settled in the bottom of the tubes with clear supernatant indicating no hemolysis.

Incubation of RBC suspension with different concentrations of NaF caused pronounced swelling. The cells remained settled at the bottom of the tube, but the saline developed a reddish colour, indicating hemolysis. The results revealed dose-dependent significant increase in hemolysis (Table 1).

**Table 1.** Hemolytic effect of sodium fluoride on RBC suspensions (Mean  $\pm$  SEM; n=20)

Concentration of NaF ( $\mu\text{g/mL}$ in final volume)	Hemolysis (%)
0.0	2.26 $\pm$ 2.29
50	6.44 $\pm$ 0.26*
100	9.45 $\pm$ 0.42*
200	14.24 $\pm$ 1.16*
300	27.94 $\pm$ 2.09*
400	74.77 $\pm$ 1.09*
500	88.90 $\pm$ 2.16*

\*Significant at the level  $P < 0.001$ .

Addition of black tea extract alone to RBC suspensions did not cause any significant effect on hemolysis. However, addition of black tea extract (2.5–100  $\mu\text{g/mL}$ ) in NaF (500  $\text{g/mL}$ ) treated tubes, reduced the rate of hemolysis (Table 2). The amelioration was dose-dependent, with maximum (92.21%) at 40  $\mu\text{g/mL}$ . A further increase in concentration of black tea extract (50–100  $\mu\text{g/mL}$ ) showed comparatively lesser rate of hemolysis, but it always remained lower than that of NaF treated tubes alone (Table 2).

**Table 2.** Percent hemolysis induced by NaF and percent reduction in hemolysis by black tea extract *in vitro* (Mean  $\pm$  SEM; n = 20)

Treatment		Percent hemolysis (%)	Reduction (%)
Sodium fluoride ( $\mu\text{g/mL}$ in final volume)	Black tea extract ( $\mu\text{g/mL}$ in final volume)		
0	0	2.70 $\pm$ 0.24	-
0	100	4.72 $\pm$ 0.95	-
500	0	71.68 $\pm$ 3.208 <sup>a*</sup>	-
500	2.5	57.41 $\pm$ 2.87 <sup>b*</sup>	19.90
500	5	18.62 $\pm$ 5.166 <sup>b*</sup>	74.02
500	10	13.18 $\pm$ 3.443 <sup>b*</sup>	81.61
500	20	11.37 $\pm$ 4.180 <sup>b*</sup>	84.13
500	30	5.88 $\pm$ 0.423 <sup>b*</sup>	91.79
500	40	5.58 $\pm$ 0.272 <sup>b*</sup>	92.21
500	50	9.57 $\pm$ 1.012 <sup>b*</sup>	86.64
500	60	23.38 $\pm$ 7.449 <sup>b*</sup>	67.38
500	70	26.56 $\pm$ 5.355 <sup>b*</sup>	62.94
500	80	27.26 $\pm$ 5.680 <sup>b*</sup>	61.96
500	90	38.40 $\pm$ 7.608 <sup>b*</sup>	46.42
500	100	39.70 $\pm$ 7.672 <sup>b*</sup>	44.61

<sup>a</sup>Compared with control; <sup>b</sup>compared with sodium fluoride control; \*significant at the level  $p < 0.001$ .

## DISCUSSION

The present results demonstrate that various concentrations of NaF in the range of 50 to 500  $\mu\text{g/mL}$  cause destabilization of RBC membrane leading to influx of water into the cells thereby causing hemolysis. However, the exact mechanism of this action is not clearly understood. Thus it might be due to an increase in lipid peroxidation and oxidative damage. F also has remarkable effects on membrane protein and membrane permeability of sperm cells.<sup>12</sup> Moreover, high F concentrations may disturb the anion channel of the erythrocyte membrane, which leads to hemolysis and swelling of cells.<sup>13-15</sup> Thus, cytotoxicity of NaF is dose-dependent.

Concurrent addition of NaF (500  $\mu\text{g/mL}$ ) and black tea extract (2.5–100  $\mu\text{g/mL}$ ) to RBC suspension caused significant reduction in the rate of hemolysis, which may be due to the presence of polyphenols in black tea extract. Various amounts of polyphenols like catechine gallate and gallocatechin gallate are found exclusively in tea.<sup>15-16</sup> In the manufacture of black tea, the 'fermentation' process causes green tea catechins to be oxidized and form oligomeric flavonols, including theaflavins, thearubigin, and other oligomers.<sup>16</sup> A review of 93 intervention studies on the bioavailability and bioefficiency of polyphenols in humans revealed that, after consumption of black tea, polyphenol levels of tea catechins in plasma increase to an effective level.<sup>16-18</sup>

Polyphenols are well known for their ability to reduce membrane lipid peroxidation and to increase the production of malondialdehyde, which can prevent oxidative damage caused by sodium fluoride. In the present work, the lack

of significant additional amelioration of hemolysis beyond 40 µg/mL of black tea extract in the RBC suspensions supports earlier findings suggesting hemolytic effects from the presence of F in tea.<sup>16</sup> Hence, black tea in high concentrations may increase the F load in erythrocytes, which might overcome its ameliorative effect. Nevertheless, the percent hemolysis in the presence of only black tea extract as compared to NaF treated suspensions was extremely low (Table 2). We conclude therefore that black tea extract significantly ameliorates F-induced hemolysis in RBC suspensions.

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