

COMET ASSAY OF DNA DAMAGE IN BRAIN CELLS OF ADULT RATS EXPOSED TO HIGH FLUORIDE AND LOW IODINE

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SUMMARY: Thirty-two one-month-old Wistar albino rats were divided randomly into four equal groups of eight (female:male = 3:1). To assess damage to DNA in their brain cells, the first group (1) of rats served as the untreated control, the second group (2) was administered high fluoride (HiF, 100 mg NaF/L in the drinking water), the third group (3) was placed on a low iodine intake (LI, 0.0855 mg I/kg diet), and the fourth group (4) was exposed to the same high fluoride and low iodine combined (HiF+LI). At 20 months of age, the rats were anesthetized and their brain cells prepared for single cell gel electrophoresis (SCGE = comet assay). DNA damage in the brain cells assessed by the tailing ratio percent was $24.68 \pm 20.81\%$ in the control group 1 and in the HiF, LI, and HiF+LI groups 2, 3, and 4, it was $90.93 \pm 9.17\%$, $89.04 \pm 4.99\%$, and $92.48 \pm 4.04\%$, respectively. Moreover, the proportion of grade III damage to the brain cells increased by 24.00% in the HiF group 2, 21.88% in the LI group 3, and 33.33% in the HiF+LI group 4, but only by 8.0% in the control group 1. These results indicate that DNA strands in the brain cells of rats are adversely affected by exposure to high fluoride, low iodine, and together in combination.

Keywords: Comet assay; DNA damage; High fluoride intake; Iodine deficiency; Rat brain cells; Single cell gel electrophoresis (SCGE).

INTRODUCTION

Epidemiological investigations reveal that the IQ of children living in high fluoride areas of Tianjin, Guizhou, and other provinces of China is 8–12% lower than in children living in low fluoride areas.^{1,2} However, these differences are greater by up to 25% in areas of both high fluoride and low iodine.³ As an archoplasm intoxicant,⁴ fluoride can induce chromosomal aberrations,⁵ sister chromatid exchange,^{5,6} and DNA damage^{4,7} in different tissues. Guan et al.⁸ found that high fluoride can penetrate the blood-brain barrier and inhibit the synthesis of DNA and RNA in the brains of offspring rats. This results in a decrease in the DNA and RNA content as well as the mass of the brain and in a slower development of it. Furthermore, the incidence of the Down syndrome congenital chromosome abnormality affecting the brains of children of younger mothers is higher in high fluoride areas.⁹

Single cell gel electrophoresis (SCGE) or comet assay is a simple, rapid, and sensitive technique for measuring DNA damage. The alkaline version of the method is a very sensitive assay procedure for the detection of single-stranded breaks in DNA.^{10,11}

In view of the results of our previous research on the effects of high fluoride and low iodine concentrations on biochemical indexes and the histopathology of

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the brain and learning-memory in offspring rats,¹²⁻¹⁴ we have now investigated SCGE to detect DNA damage in brain cells of adult rats exposed to high fluoride and low iodine for an extended period of time.

MATERIALS AND METHODS

Experimental protocol: As in our recent reports,¹²⁻¹⁴ one-month old Wistar albino rats, each weighing approximately 50 g, were obtained from the Experimental Animal Center of Shanxi Medical University for use in this study.

The same iodine-deficient feed and high-fluoride water reported in our previous studies¹²⁻¹⁴ were also employed here as shown in Table 1.

Table 1. Fluoride in the drinking water (mg F⁻/L) and fluoride and iodine levels in the diet (mg/kg) of the rats

	Control	High fluoride (HiF)	Low iodine (LI)	High fluoride and low iodine (HFLI)
Iodine in diet	0.3543	0.3543	0.0855	0.0855
Fluoride in diet	25.57	25.57	26.01	26.01
Fluoride in drinking water	<0.6	45 ^a	<0.6	45 ^a

^aFrom 100 mg NaF/L (as recorded in our three previous reports¹²⁻¹⁴).

Animal test model: Thirty-two one-month old Wistar albino rats (female:male = 3:1) were randomly divided into four groups of six females and two males each and were maintained on the diets and water regimens shown in Table 1 under standard temperature (22–25°C), ventilation, and hygienic conditions. When the rats were 20 months old, they were sacrificed as described below for testing of their brain cells by single cell gel electrophoresis (SCGE). The number of rats in each experimental group was as follows: five controls (female:male = 4:1), five in the HiF group (female:male = 4:1), five in the LI group (female:male = 3:2), and six in the HiF + LI group (female:male = 5:1).

Preparation of single cell suspensions: After the rats were anesthetized with 20% urethane (ethyl carbamate, NH₂COOC₂H₅) solution, they were sacrificed by transcardial perfusion with 0.9% saline. The brains were then removed, washed three times with phosphate buffer solution (PBS: NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄·12H₂O 2.8 g, KH₂PO₄ 0.2 g, pH 7.4), cut into pieces with stainless steel scissors, homogenized with the appropriate amount of PBS at 4°C and pH 7.4 in a glass homogenizer at 0–4°C, and then sifted through a 300-μm sieve. The slides were previously stained by trypan blue (3,3'-[3,3'-Dimethyl(1,1'-biphenyl)-4,4'-diyl]bis(azo)}bis-(5-amino-4-hydroxy)-2,7-naphthalenedisulfonic acid, tetra-sodium salt), and under a microscope up to 90% of the brain cells were found to be alive. The cells were resuspended at approximately 10⁶ per mL in PBS and then used immediately for comet assay.

Alkaline comet assay (single cell gel electrophoresis): Alkaline comet assay was performed essentially according to the published procedure with some

modifications.¹⁰ At least 100 cells per slide per subject were analyzed (original magnification $\times 200$) under a fluorescent microscope (BX51, Olympus) equipped with a green light excitation and at 590-nm barrier filter. Comets form as the broken ends of a negatively charged DNA molecule becomes free to migrate in the electric field toward the anode. The ratio of tailing was assessed by counting the tailing DNA in 100 cells per sample. Twenty-five cells were chosen randomly and photographed to measure the length of DNA migration and to grade the cells in each sample. The extent of DNA damage was assessed from the length of DNA migration derived by subtracting the diameter of the nucleus from the total length of the image. Grading was as follows: Grade I: tailing length/diameter of the nucleus <1 ; grade II: tailing length/diameter of the nucleus <2 ; grade III: tailing length/diameter of the nucleus $=2$. Grades I and II indicate generic rupture of the DNA chain. Grade III indicates apoptosis, presenting a small comet head and a large and bright tail that it looks like a broom.

RESULTS

In comparison with the controls, the ratios of tailing and tail length of brain cells exposed to high fluoride, low iodine, and the combination of high fluoride and low iodine increased considerably (see Tables 2 and 3), and experimental cells show the typical comet configurations seen in Figures 1–4. The proportion of grade III damage to brain cells increased considerably, as shown in the frequency histogram (Figure 5).

Table 2. Ratio of tailing in brain cells induced by high fluoride and low iodine (Mean \pm SD)

	No. of samples	No. of cells analyzed	Ratio of tailing (%)
Control	5	500	24.68 \pm 20.81
Low iodine (LI)	5	500	89.04 \pm 4.99*
High fluoride (HiF)	5	500	90.93 \pm 9.17*
High fluoride and low iodine (HiF+LI)	6	600	92.48 \pm 4.04*

* $p < 0.01$.

Table 3. Length of comet tail in brain cells induced by high fluoride and low iodine (Mean \pm SD)

	No. of cells analyzed	Length of tail (μ m)
Control	125	10.46 \pm 12.53
Low iodine (LI)	125	18.41 \pm 10.06*
High fluoride (HiF)	125	25.57 \pm 17.25†
High fluoride and low iodine (HiF+LI)	150	27.31 \pm 15.02†

* $p < 0.05$; † $p < 0.01$.

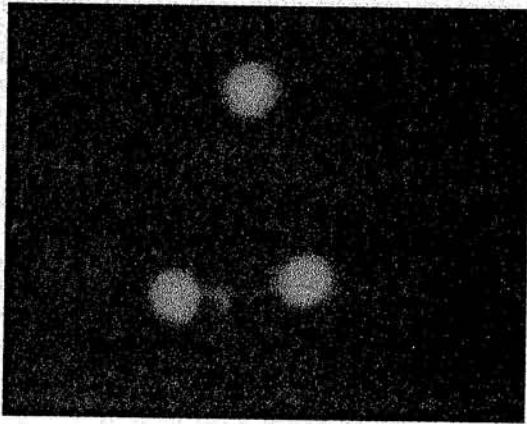


Figure 1. Comet of brain cells in controls.

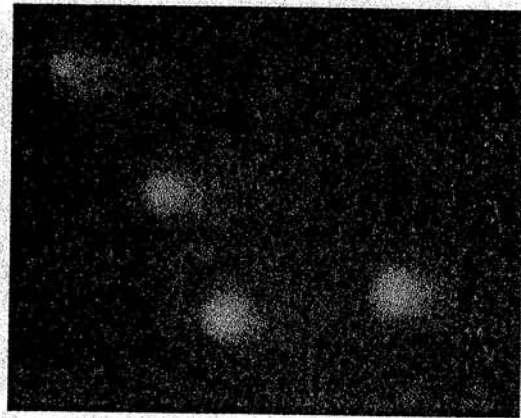


Figure 2. Comet of brain cells in LI group.



Figure 3. Comet of brain cells in HiF group.

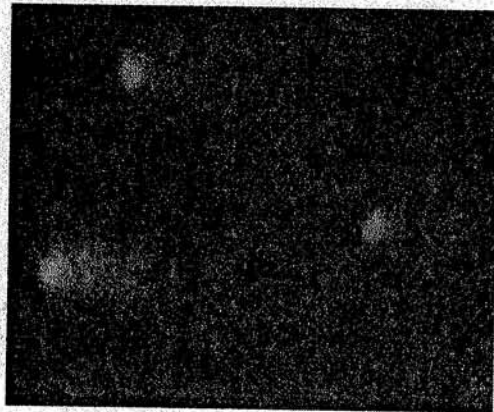


Figure 4. Comet of brain cells in HiF+LI group.

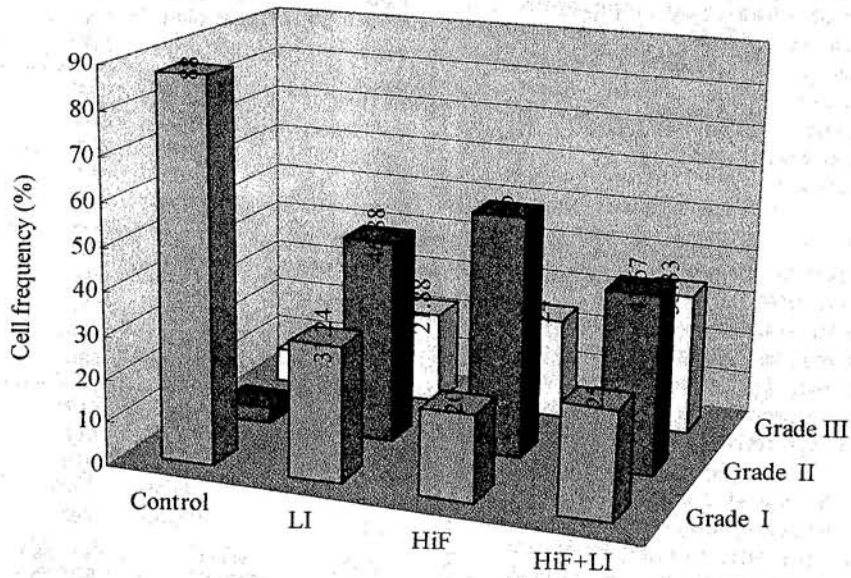


Figure 5. Frequency histogram of undamaged and migrated brain cells in control and LI, HiF, and HiF+LI rats.

DISCUSSION

Brain damage induced in adult rats In this study, the percentage of damaged brain cells of grades II and III was up to 12% higher than in the control group, and the ratio of tailing was 24.68%. On the other hand, in young Sprague-Dawley rat brains, Chen et al.¹⁵ found that the total ratio of brain cell damage was only 7.75%. Since the structure and function of organisms gradually deteriorates with time, age is therefore a possible factor that induces DNA damage in cells. Moreover, free radical concentrations *in vivo* also tend to increase with age, whereas the capacity to combat free radicals gradually decreases, resulting in increasing accumulation of free radicals. Increased free radical concentrations lead to undesirable oxidations of many chemicals, alterations in enzyme activity, and increased rates of apoptosis and DNA damage.

DNA damage induced by high fluoride and low iodine in rat brains: DNA damage of brain cells exposed to high fluoride, low iodine, and their combined interaction markedly increased, especially in the HiF+LI group. Fluoride not only penetrates the blood-brain barrier, but it can also induce DNA damage in brain cells. A possible mechanism of DNA damage induced by fluoride is as follows: (1) Fluoride has a dense negative charge and is biochemically very active. Thus it can also have a direct effect on DNA because of its strong affinity for uracil and amide bonds by $-NH\cdots F^-$ interactions that can induce the rupture of hydrogen bonds in the base pairing of adenine and thiamine, resulting in disturbance of the synthesis of DNA and RNA and increasing error frequency of linkage between basic groups in the process of DNA replication.¹⁶ In addition, some studies show that fluoride can combine stably with DNA by covalent bonding, affecting the normal structure of DNA.¹⁷ (2) Fluoride can induce the production of free radicals, which can damage DNA strands directly or by lipid peroxidation initiated by free radicals.¹⁸ (3) Fluoride may depress enzyme activity, such as with DNA polymerase.¹⁹ This might further affect the process of DNA replication or repair and thereby damage DNA.

Thyroid deficiency in early life has a marked influence on the functional development of the central nervous system and is accompanied by significant effects on the structural and biochemical maturation of the cerebellum.²⁰ In this study, the rate and degree of DNA damage of brain cell in aged rats is higher in the HiF+LI group than in the other experimental groups. This suggests that a low iodine intake coupled with exposure to high fluoride exacerbates lesions in the central nervous system.³ Because our results are a snapshot of adult rat development without dynamic observations of time-trends, the extent of DNA damage from exposure to fluoride in combination with iodine deficiency at various stages of development obviously deserves further study.

In conclusion, DNA strands in brain cell are affected adversely when rats are exposed to high fluoride, low iodine, and the interactive combination of these two factors from the age of one month to 20 months. The rate and degree of DNA damage in brain cells is higher in the HiF+LI group than in either the HiF or LI group.

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