Effect of Vitamin C against the Genotoxicity Induced by N-Nitrosodiethanolamine in Mice *in vivo* Test System

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Summary N-Nitrosodiethanolamine (NDELA) constitutes a serious potential genotoxic and carcinogenic hazard. The mutagenic potential of NDELA was evaluated in mice *in vivo* using the 2 cytogenetic parameters, chromosomal aberration and sister chromatid exchange. Single oral treatment by gavage using different doses of NDELA 10, 20, 50, 100, 250, 400 mg kg⁻¹ b.wt. induced an increase in the percentage of chromosomal aberrations in mouse bone-marrow as well as in mouse primary spermatocytes in a dose-dependent relationship. Such percentage was statistically significant at the doses ranging from 20–400 mg. Repeated dose treatment with the doses 50, 100, 250 mg kg⁻¹ b.wt. NDELA for 3 consecutive days induced slight increase in the incidence of chromosomal aberrations over that of a single dose treatment. The results also show that NDELA is a potent inducer of sister chromatid exchanges (SCE's). Intraperitoneal treatment with the doses 100, 250, 400 NDELA induced a significant and dose dependent increase in the frequency of SCE's. Such frequency reached $14.90 \pm 0.68/\text{cell}$ and $18.20 \pm 0.48/\text{cell}$ ($p < 0.01$) after treatment with the 2 higher doses 250 and 400 mg kg⁻¹ b.wt. which exceed the corresponding value of mitomycin C (13.90 ± 0.63) as the positive control. Concurrent administration of vitamin C (L-ascorbic acid) at a dose of 20 mg kg⁻¹ b.wt. moderately minimized the genotoxic effects of NDELA in all experiments examined.

N-nitrosodiethanolamine is among the most widely distributed of the N-nitroso compounds and probably the one to which human exposure is the greatest (Scanlan 1983, Lijinsky and Kovatch 1985), since it occurs at high levels (up to 3%) in cutting and grinding fluids in metal industries (Fan *et al.* 1977b, Spiegelhalder and Preussmann 1984, Spiegelhalder *et al.* 1987), in cosmetics, lotions, shampoos (Fan *et al.* 1977a), tobacco and pesticides (IARC Monographs 1978). NDELA can be readily formed by interaction between sodium nitrite and diethanolamine or triethanolamine (Zingmark and Rappe 1976).

NDELA is a strong animal carcinogen (Druckrey *et al.* 1967, Hilfrick *et al.* 1977, Preussmann *et al.* 1982), a mutagen in the Ames test after activation with alcohol dehydrogenase (Denkel *et al.* 1986, Eisenbrand *et al.* 1984) and a potent inducer of DNA damage in primary hepatocytes *in vitro* (Sina *et al.* 1983).

It is very important, nowadays to search for protective substances that could minimize the toxic effects of different chemicals. However, vitamins play a beneficial roll against the mutagenicity of some chemicals (Odin 1997). Vitamin C successfully reduced the clastogenic effects of many chemical agents and radiation *in vivo* assays (Hoda and Sinha 1991, Ghaskadbi *et al.* 1992).

Therefore, the present study was undertaken to evaluate the mutagenic potential of N-nitrosodiethanolamine in mice *in vivo* test systems and to test the antigenotoxic efficiency of vitamin C in a trial to minimize the genotoxicity of the N-nitroso compounds.

Materials and methods

Animals

Mature male swiss mice 9–12 weeks old, weighing 25–30 g were used in all experiments. The animals were obtained from a closed random bred colony at the National Research Centre, main-

tained under controlled conditions of temperature and humidity and received food and water *ad libitum*.

Chemicals

N-nitrosodiethanolamine $C_4H_{10}N_2O_3$ (Sigma Chemical) N-7632.

Treatment and cytological preparation

Chromosomal aberration For bone-marrow or spermatocytes, groups of mice (5/dose) were orally treated by gavage with a single dose of 10, 20, 50, 100, 250, 400 mg kg⁻¹ b.wt. NDELA dissolved in distilled water. Another groups were orally treated with a single dose of NDELA at 50, 100, 250, 400 mg kg⁻¹ b.wt. and with vitamin C at 20 mg kg⁻¹ b.wt. (concurrent administration). Samples were taken 24 h post treatment. For repeated dose treatment, mice were orally treated with 3 successive daily doses of NDELA at the dose levels 50, 100, 250 mg kg⁻¹ b.wt. Another groups treated with the same doses of NDELA in addition to vitamin C 20 mg kg⁻¹ b.wt. dissolved in distilled water (concurrent administration). Samples were taken 24 h after the last treatment.

Chromosomes from bone-marrow cells were prepared following the method of Yosida and Amano (1965). Spermatocyte metaphases at diakinesis metaphase I was prepared according to Evans *et al.* (1964). Slides were stained with 7% Giemsa in phosphate buffer pH 6.8. At least 75–100 well spread metaphases were analyzed for each animal scoring different types of aberrations.

Sister chromatid exchange The method described by Allen (1982) for conducting *in vivo* SCE induction analysis in mice was applied with modification. 5-Bromodeoxyuridine (BrdU, Sigma) tablets weighing 55 ± 5 mg were placed subcutaneously for 21 h. Mice were injected intraperitoneally with NDELA at the doses of 100, 250, 400 mg kg⁻¹ b.wt. and also with NDELA at the same concentrations in addition to vitamin C 20 mg kg⁻¹ b.wt. (concurrent administration) 8 h following BrdU treatment. Mice were injected with colchicine (0.6 mg/kg) 2 h prior to killing. Bone-marrow cells were fixed in methanol–acetic acid (3:1) and stained with fluorescence plus Giemsa method of Goto *et al.* (1978). Five animals were taken for each treatment and 40 well-spread metaphases per animal were examined microscopically for SCE's.

In all experiments an untreated group acted as control and mitomycin C at a dose of 1 mg kg⁻¹ b.wt. (i.p.) was used as a positive control. The third control group was treated orally with vitamin C 20 mg kg⁻¹ b.wt.

Statistical analysis The significance of the experimental versus control data was calculated using the t-test.

Results and discussion

The mutagenic potential of N-nitrosodiethanolamine was evaluated *in vivo* using the two cytogenetic parameters, chromosomal aberration and sister chromatid exchange. *In vivo* genetic toxicity testing of a compound allows the detection of abnormalities produced by the test compound or its metabolites (Sinha *et al.* 1983, Krishna *et al.* 1985).

The present results show that a single oral treatment (gavage) with NDELA induced an increase in the percentage of chromosomal aberrations in bone-marrow cells at the tested concentrations 10, 20, 50, 100, 250, 400 mg kg⁻¹ b.wt. in a dose-dependent relationship (Table 1). Such percentage was statistically significant at the doses ranging from 20–400 mg even after excluding the number of metaphases with chromatid or chromosome gaps. The percentage of the induced aberrations reached 15.28 ± 0.62 ($p < 0.01$) after single oral treatment with the highest tested dose which was greatly lower than that value induced by the positive control mitomycin C (29.40 ± 0.51). Repeated dose treatment with the doses 50, 100, 250 mg kg⁻¹ b.wt. for 3 consecutive daily doses in-

Table 1. Frequency of chromosomal aberrations in mouse bone-marrow cells after single oral treatment with different concentrations of N-nitrosodietanolamine

Treatment and doses	Total number of examined metaphases H ^o (n)	No. of abnormal metaphases	No. of metaphases with ^a					Mean % aberrant metaphases $\pm$ S.E.		
			Gap	Frag.	Break	Del.	RT	MA	Polyp.	Including gaps
I. Control (non treated)	400	14	9	2	—	3	—	—	3.50 $\pm$ 0.29	1.25 $\pm$ 0.40
II. N-nitrosodietanolamine										
10 mg kg ⁻¹ b.wt.	400	19	9	1	2	3	1	1	2	4.75 $\pm$ 0.48
20 mg kg ⁻¹ b.wt.	400	26	10	2	3	6	2	2	1	6.50 $\pm$ 0.29*
50 mg kg ⁻¹ b.wt.	400	33	14	3	3	7	2	2	2	8.25 $\pm$ 0.48**
100 mg kg ⁻¹ b.wt.	400	45	16	6	3	12	3	2	3	11.25 $\pm$ 0.85**
250 mg kg ⁻¹ b.wt.	400	56	19	11	5	14	2	3	2	14.00 $\pm$ 1.15**
400 mg kg ⁻¹ b.wt.	700	107	36	13	11	30	3	9	5	15.28 $\pm$ 0.62**
III. Vitamin C (VC)										
20 mg kg ⁻¹ b.wt.	500	21	8	7	—	2	2	—	2	4.20 $\pm$ 0.37
IV. N-nitrosodietanolamine + VC (20 mg)										
100 mg + VC	400	33	8	7	4	7	3	2	2	8.25 $\pm$ 0.63**
250 mg + VC	400	42	12	8	5	7	5	2	3	10.50 $\pm$ 0.97**
400 mg + VC	400	55	14	11	7	10	5	3	5	13.75 $\pm$ 0.94**
V. Mitomycin C (1 mg kg ⁻¹ b.wt.) positive control	500	147	39	22	18	22	8	33	5	29.40 $\pm$ 0.51
										21.60 $\pm$ 0.66**

H^o = Harvest time after the last treatment = 24 h, (n) = Number of animals per each treatment group = 4 or 5, ^a Frag. = Fragment, Del. = Deletion, RT = Rebertsonian translocation, MA = metaphases contain more than one type of chromosomal aberrations, Polyp. = Polyploidy, * Significant at 0.05 level, ** Significant at 0.01 level (t-test).

Table 2. Frequency of chromosomal aberrations in mouse bone-marrow cells induced after repeated oral treatment with different concentrations of N-nitrosodiethanolamine

Treatment and doses	Total number of examined metaphases H° (n)	No. of abnormal metaphases	No. of metaphases with ^a						Mean % aberrant metaphases ± S.E.	
			Gap	Frag.	Break	Del.	RT	MA	Polyp.	Including gaps Excluding gaps
I. Control (non treated)	400	13	9	1	1	2	—	—	—	1.00 ± 0.60
II. N-nitrosodiethanolamine										
50 mg kg ⁻¹ b.wt.	400	37	10	12	7	5	1	2	—	6.75 ± 0.50**
100 mg kg ⁻¹ b.wt.	400	51	12	11	10	8	3	3	4	9.75 ± 0.63**
250 mg kg ⁻¹ b.wt.	400	65	16	14	16	11	5	3	—	12.25 ± 0.48**
III. Vitamin C (V.C)										
20 mg kg ⁻¹ b.wt.	400	19	10	5	1	2	—	—	1	2.25 ± 0.30
IV. N-nitrosodiethanolamine + V.C (20 mg)										
50 mg + V.C	400	31	8	9	6	6	1	1	—	5.75 ± 0.58*
100 mg + V.C	400	44	13	10	8	6	4	3	—	7.75 ± 0.82**
250 mg + V.C	400	51	16	10	11	7	5	2	—	8.75 ± 0.75**

H° = Harvest time after the last treatment = 24 h, (n) = Number of animals per each treatment group = 4 or 5, ^a (See Table 1), * Significant at 0.05 level, ** Significant at 0.01 level (t-test).

Fig. 1. Metaphases from mouse treated with different doses of NDELA showing a) chromatid gap, b) chromosome gap, in mouse bone marrow cells. c) X-Y univalent, d) autosomal univalent, e) frag., f) break, g) chain III+1, in mouse spermatocytes. h, i) Metaphases from mouse bone marrow showing, SCE's induced after treatment with NDELA.

duced slight increase in the incidence of chromosomal aberrations over that of the single dose treatment (Table 2). This may be explained by the fact that 60–90% of NDELA is excreted unchanged in the urine within 24 h (Monarco *et al.* 1996).

With respect to the types of the induced aberrations, the numerical chromosomal aberrations were rare, while the structural types had significant values and were observed in the form of gaps (Fig. 1a), breaks (Fig. 1b), fragments, deletions, and translocations. The majority of aberrations are of chromatid type. Chromosomal aberrations and formation of micronuclei were also shown in human lymphocytes after incubation with NDELA *in vitro* (Dittberner *et al.* 1988). NDELA was found to induce DNA damage in mononuclear blood cells of metal workers depending on its concentration (Fuchs *et al.* 1995). DNA single strand breaks were detected in rat hepatocytes after incubation with NDELA *in vitro* (Schmezer *et al.* 1987) as well as in rat liver cells after oral application of NDELA *in vivo* (Denkel *et al.* 1986, Sterzel and Eisenbrand 1986).

Referring to the mechanism of action of nitroso compounds as mutagen or carcinogen, it was reported that nitrogen mustards (which are mutagenic and carcinogenic) react with nucleic acids resulting in alkylation principally of guanylic acid moieties at the 7-position. This alkylation of DNA is responsible for mutagenesis by the compounds (Hollaender 1976, Singer 1979).

Some vitamins may scavenge harmful species, free radicals or electrophiles, which damage DNA and other cell targets (Odin 1997). Vitamin C successfully reduced the clastogenic effects of many chemical agents and radiation *in vivo* assay (Hoda and Sinha 1991, Ghaskadbi *et al.* 1992, Sarma and Kesavan 1993, Khan and Sinha 1996). Moreover, ascorbic acid has been reported to in-

Table 3. Frequency of chromosomal aberrations in primary spermatocytes of male mice induced after single oral treatment with different concentrations of N-nitrosodiethanolamine

Treatment and doses	Total number of examined metaphases H° (n)	No. of abnormal metaphases	Mean % abnormal metaphases ± S.E.	No. of metaphases with different types of chromosomal aberrations ^a							
				x-y	u	A-u	Gap	Frag.	Break	Chain	MA
Single dose treatment											
I. Control (non treated)	400	18	4.50±0.80	11	7	—	—	—	—	—	—
II. N-nitrosodiethanolamine											
10 mg kg ⁻¹ b.wt.	400	21	5.25±0.75	11	8	—	—	2	—	—	—
20 mg kg ⁻¹ b.wt.	450	30	6.66±0.60*	15	10	—	—	2	3	—	—
50 mg kg ⁻¹ b.wt.	400	37	9.25±0.48**	17	12	2	2	3	3	—	—
100 mg kg ⁻¹ b.wt.	400	46	11.50±0.64**	20	18	1	1	3	4	—	—
250 mg kg ⁻¹ b.wt.	400	54	13.50±0.54**	20	17	3	3	4	9	1	—
400 mg kg ⁻¹ b.wt.	400	85	21.25±0.95**	24	28	3	3	5	18	4	3
III. Vitamin C (VC)											
(20 mg kg ⁻¹ b.wt.)	400	19	4.75±0.59	12	7	—	—	—	—	—	—
IV. N-nitrosodiethanolamine+ VC (20 mg)											
100 mg+ VC	400	32	8.00±0.65**	18	11	—	—	1	2	—	—
250 mg+ VC	400	43	10.75±0.71**	24	12	2	2	2	3	—	—
400 mg+ VC	400	63	15.75±0.70**	25	17	2	2	2	10	3	4
V. Mitomycin C											
1 mg kg ⁻¹ b.wt. positive control	500	107	21.40±0.85**	42	27	6	6	9	10	2	11

H^o = Harvest time after the last treatment = 24 h, (n) = Number of animals per each treatment group = 4 or 5, ^ax-y u = x-y univalent, A-u = Autosomal univalent, Frag. = Fragment, MA = Metaphases contain more than one type of chromosomal aberrations, * Significant at 0.05 level, ** Significant at 0.01 level (t-test).

Table 4. Frequency of chromosomal aberrations in primary spermatocytes of male mice induced after repeated oral treatment with different concentrations of N-nitrosodiethanolamine

Treatment and doses	Total number of examined metaphases H ^o (n)	No. of abnormal metaphases	Mean % abnormal metaphases $\pm$ S.E.	No. of metaphases with different types of chromosomal aberrations ^a						
				x-y u	A-u	x-y u + A-u	Gap	Frag.	Break	Chain
Repeated dose treatment 3 successive dose treatment										
I. Control										
(non treated)	400	16	4.00 $\pm$ 0.25	10	6	—	—	—	—	—
II. N-nitrosodiethanolamine										
50 mg kg ⁻¹ b.wt.	400	41	10.25 $\pm$ 0.48**	20	13	—	2	2	4	—
100 mg kg ⁻¹ b.wt.	400	52	13.00 $\pm$ 0.75**	21	17	4	2	3	5	—
250 mg kg ⁻¹ b.wt.	400	61	15.25 $\pm$ 0.69**	22	16	5	3	3	11	1
III. Vitamin C (V.C)										
(20 mg kg ⁻¹ b.wt.)	400	20	5.00 $\pm$ 0.48	13	6	—	1	—	—	—
IV. N-nitrosodiethanolamine+V.C (20 mg)										
50 mg kg ⁻¹ b.wt. + V.C	400	34	8.50 $\pm$ 0.50**	18	12	—	1	1	2	—
100 mg kg ⁻¹ b.wt. + V.C	400	42	10.50 $\pm$ 0.73**	18	14	3	1	2	4	—
250 mg kg ⁻¹ b.wt. + V.C	400	51	12.75 $\pm$ 0.52**	23	14	3	2	2	7	—

H^o = Harvest time after the last treatment = 24 h, (n) = Number of animals per each treatment group = 4 or 5, ^a x-y u = x-y univalent, A-u = Autosomal univalent, Frag. = Fragment, * Significant at 0.05 level, ** Significant at 0.01 level (t-test).

hibit the formation of carcinogenic N-nitroso compounds from precursors by reacting with nitrite to form nitrosoascorbate, thus competing with amines for available nitrosylating anions (Bogovski *et al.* 1972, Mirvish *et al.* 1972). The present results (Tables 1, 2) show that vitamin C 20 mg kg⁻¹ b.wt. (double the human therapeutic dose) did not induce chromosomal abnormalities of its own. Oral administration of vitamin C to mice treated with NDELA (concurrent administration) slightly minimized the percentage of chromosomal abnormalities in both single and repeated treatment. The mean percentage of aberrant metaphases reached 12.75±0.67 in mouse bone-marrow after repeated dose treatment (3 successive daily doses) with 250 mg NDELA and vitamin C compared with 16.25±1.10 for those treated with NDELA alone (Table 2).

NDELA also induced chromosomal abnormalities in mouse primary spermatocytes at diakinesis metaphase I after single and repeated treatment (Tables 3, 4). The effect was dependent on NDELA concentration. The mean percentage of aberrations reached 21.25±0.95 (p<0.01) after single oral treatment with the highest tested dose 400 mg kg⁻¹ b.wt. while it reached 21.40±0.85 for the positive control mitomycin C. The high incidence of aberrant spermatocytes definitely indicates the mutagenicity of NDELA with the high doses. Such effect on germ cells may be transmitted to the following generations (William and Hsu 1980, Hassan 1997). The types of the induced aberrations recorded (Fig. 1c-g) were x-y univalents, autosomal univalents, gaps, fragments, breaks, translocations (chain III and chain IV). Concurrent administration of vitamin C (as single or repeated treatment) slightly minimized the incidence of chromosomal abnormalities induced by NDELA in primary spermatocytes.

The present results also show that NDELA is a potent inducer of SCE's (Table 5, Fig. 1h, i). However SCE is a well-known biomarker of exposure to mutagen/carcinogen, a large number of chromosome damaging agents has been shown to induce SCE's (Wilkosky and Rynard 1990). Alkylating agents such as nitrosamine have been shown to produce SCE's *in vitro* (Wilkosky and Rynard 1990). Intraperitoneal treatment with NDELA at the doses of 100, 250, 400 mg kg⁻¹ b.wt. induced a significant and a dose-dependent increase in the frequency of SCE's. The frequency of SCE's reached 14.90±0.68/cell and 18.20±0.48/cell (p<0.01) after treatment with the two higher doses 250 and 400 mg kg⁻¹ b.wt, respectively which exceed significantly the corresponding value of mitomycin C 13.90±0.63/cell as the positive control. It has been established that mitomycin C is

Table 5. Frequency of sister chromatid exchanges (SCE's) in mouse bone-marrow cells induced after i.p. treatment with different concentrations of N-nitrosodiethanolamine

Treatment and doses	Mice No.	Metaphases with SCE's No.	SCE's No.	SCE's/Cell Mean±S.E.
I. Control (non treated)	5	200	1240	6.20±0.48
II. N-nitrosodiethanolamine				
100 mg kg ⁻¹ b.wt.	7	280	2313	8.26±0.17*
250 mg kg ⁻¹ b.wt.	5	200	2980	14.90±0.68**
400 mg kg ⁻¹ b.wt.	5	200	3640	18.20±0.48**
III. Vitamin C (V.C) (20 mg kg ⁻¹ b.wt.)	7	250	1675	6.70±0.43
IV. N-nitrosodiethanolamine + Vitamin C (20 mg kg ⁻¹ b.wt.)				
100 mg kg ⁻¹ b.wt. + V.C	6	240	1824	7.60±0.25
250 mg kg ⁻¹ b.wt. + V.C	5	200	2606	13.03±0.28**
400 mg kg ⁻¹ b.wt. + V.C	5	200	3460	17.30±0.35**
V. Mitomycin C 1 mg kg ⁻¹ b.wt. (positive control)	5	200	2780	13.90±0.63

* Significant at 0.05 level, ** Significant at 0.01 level (t-test).

mutagenic (Carrano *et al.* 1978). Mitomycin C is one of the most powerful inducers of SCE's (Russo *et al.* 1993). The high incidence of SCE's that induced after treatment with NDELA may reflect its action on the cellular DNA repair process. It is known to alkylate DNA (Hollaender 1976). NDELA was previously reported to increase the incidence of SCE's in human lymphocytes *in vitro* (Henn *et al.* 1989). The concurrent administration of vitamin C (20 mg kg⁻¹ b.wt.) as single intraperitoneal treatment moderately reduced the incidence of SCE's induced by various doses of NDELA (range 0.9–1.5%). It is worth mentioning in this respect that vitamin A was previously succeeded in minimizing NDELA induced DNA damage *in vitro* in *Escherichia coli* K-12 cells (Knas-muller *et al.* 1992). On the other hand the same authors reported a moderate antigenotoxic effects of vitamin A *in vivo* biochemical assay in mice. They explained this by the fact that vitamin A appeared not to interfere strongly with alpha-hydroxylation of nitrosamine (metabolic activation step).

In conclusion, the present investigation clearly reveal the genotoxic property of N-nitrosodiethanolamine in mouse *in vivo* test system. Moderate antigenotoxic effect was obtained after using vitamin C. Much more work should be done in this respect.

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