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Genotoxic evaluation of the non-halogenated disinfection by-products nitrosodimethylamine and nitrosodiethylamine

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ABSTRACT

Disinfection by-products (DBPs) are chemicals that are produced as a result of chlorine being added to water for disinfection. As well as the halogenated DBPs, *N*-nitrosamines have recently been identified as DBPs, especially when amines and ammonia ions are present in raw water. In this work, the genotoxicity of two nitrosamines, namely nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA), has been studied in cultured human cells. To evaluate their genotoxic potential two assays were used, the comet assay and the micronucleus test. The comet assay measures the induction of single and double-strand breaks, and also reveals the induced oxidative DNA damage by using endoIII and FPG enzymes. Chromosomal damage was evaluated by means of the cytokinesis-blocked micronucleus test. The results of the comet assay show that both compounds are slightly genotoxic but only at high concentrations, NDEA being more effective than NDMA. Enzyme treatments revealed that only NDEA was able to produce increased levels of oxidized bases, mainly in purine sites. The results obtained in the micronucleus assay, which measures the capacity of the tested agents to induce clastogenic and/or aneugenic effects, are negative for both of the nitrosamines evaluated, either using TK6 cells or human peripheral blood lymphocytes. Taking into account the very high concentrations needed to produce DNA damage, our data suggest a low, if existent, genotoxic risk associated with the presence of these compounds in drinking water.

1. Introduction

A significant development relating human health has been the discovery that disinfecting water drastically decreased human deaths caused by water-borne diseases. According to Freese and Nozaic [1], chlorine disinfection of water has been credited with saving a vast number of lives worldwide on a daily basis. However, besides eliminating water pathogens, the chlorination process also leads to the formation of several compounds with genotoxic potential. These chemicals, known as disinfection by-products (DBPs), originate from the reaction of chlorine with organic or inorganic natural matter present in raw water. An important aspect to take into account is that each water treatment plant has its unique profile of DBPs, depending on factors such as the quality of raw water, the type and amount of disinfectants applied the use of secondary disinfectants, and the pH and temperature conditions, among others [2–6].

In 1974, Rook [7] described trihalomethanes (THMs) as the first class of DBPs identified in treated water, and Bellar et al. [8] determined the increasing levels of chloroform, the most frequent THM present in disinfected water. Regarding DBPs, it is of considerable concern that although more than 600 DBPs have already been reported [9], only a small number have in fact been quantified or evaluated for their genotoxic or mutagenic potential, and for their possible adverse health effects.

One class of non-halogenated DBPs is made up of nitrosamines, which have been recently identified as by-products in chlorinated drinking water [10,11], as well as in chlorinated pools [12]. *N*-nitrosamines are alkylating agents characterized by the presence of the *N*-nitroso group and may be aliphatic or ring structures. They are considered an important class of environmental non-halogenated mutagens and carcinogens and, according to the weight-of-evidence characterization, they have been included in the group B2 by the U.S. EPA [13] (http://www.epa.gov/iris/subst/0045.htm). This means that, on the basis of the induction of tumors at different sites in both rodents and non-rodent mammals exposed to nitrosamines by various routes, these compounds are probably

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carcinogenic in humans. One important feature of nitrosamines is that their carcinogenic properties decrease as the length of the aliphatic chain increases. The single exception is nitrosodiethylamine (NDEA), which has a higher carcinogenic potency than nitrosodimethylamine (NDMA) [14]. It must be pointed out that, while nitrosamines were only recently identified as by-products in chlorinated drinking water, considerable toxicological research on this chemical class over 30 years has yielded important discoveries regarding carcinogenesis. Currently, several jurisdictions have implemented regulations that require widespread measurements of NDMA and NDEA in both raw and drinking water.

NDMA is formed in the chlorination process when raw water contains dimethylamine and other secondary amines, and its concentration is closely related to the ratio of chlorine, ammonia ions and dimethylamine [10,15]. NDMA and other nitrosamines can be continuously formed in the water distribution systems and, in this way, higher levels can be detected in the distribution system than in the treatment plant. Thus, nitrosamine measures at the treatment plant may underestimate the actual human exposure [16]. According to the U.S. EPA, the maximum admissible levels of NDMA and NDEA in water were established at very low concentrations as 7 and 2 ng/L, respectively.

In this context, the aim of the present work is to provide more information on the genotoxic activity of NDMA and NDEA, which can be considered as emerging contaminants, in cultured human cells. To reach this objective, two different genotoxicity assays have been used. One is the single-cell gel electrophoresis (SCGE) assay or comet assay, which is a rapid, relatively simple and sensitive technique for measuring primary DNA damage [17]. Besides detecting single and double DNA strand breaks, this technique can also detect incomplete excision repair and alkali-labile sites. It must be emphasized that due to its high sensitivity it is able to detect DNA damage in frequencies of less than 1 damaged base in 10^7 bases [18].

In addition, the comet assay also allows the analysis of oxidative DNA damage, when the bacterial repair enzymes formamidopyrimidine DNA glycosilase (FPG) and endonuclease III (endoIII) are used. FPG and endoIII detect and break the DNA at sites with oxidized forms of purines and pyrimidines, respectively [19].

The other assay used is the micronucleus (MN) test. This assay enables other kinds of damage to be detected, such as chromosome breaks and aneuploidy [20,21], both events being of great relevance in carcinogenesis. In addition, as has been demonstrated in a recent study, the MN assay can be used as a surrogate biomarker of cancer risk [22].

We feel that the application of the two selected experimental approaches enables us to report new information on the DNA damage induced in human cells by the two nitrosamines tested (NDMA and NDEA).

2. Materials and methods

2.1. TK6 and lymphocyte cultures

The human lymphoblastoid TK6 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This cell line has been used extensively for mutagenicity and other genotoxicity studies, including both the comet and the micronucleus assays [23,24].

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood in 4.5 mL RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Chemicals

Nitrosodimethylamine (NDMA, $(CH_3)_2NNO$, CAS 62-75-9) and nitrosodiethylamine (NDEA, $(C_2H_5)_2NNO$, CAS 55-18-5) were obtained from Sigma–Aldrich (St Louis, MO, USA). Endonuclease III (endoIII) and formamidopyrimidine glycosylase (FPG) enzymes were kindly provided by Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway).

2.3. S9 fraction

The metabolic activation fraction used was S9 from male Sprague-Dawley rats induced with Aracolor 1254 (ICN Biomedicals Inc., Aurora, OH, USA). The S9 mix freshly prepared, consisted of 1 mL of S9, 0.33 mL of 1 M KCl, 0.32 mL of 0.25 M $MgCl_2 \cdot 6H_2O$, 0.25 mL of 0.2 M glucose-6-phosphate, 1 mL of 0.04 M NADP, 2.10 mL of distilled water and 5 mL of phosphate buffer (pH 7.4) [25].

2.4. Alkaline comet assay

TK6 cell cultures were centrifuged at 500 g for 2 min and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 mL). Each disinfection by-product was dissolved in distilled water and 5 concentrations of each DBP were evaluated, with and without S9 mix, simultaneously. The concentrations used were 0.05, 0.1, 1, 5 and 10 mM, for both compounds. Aliquots of 10 μL of H_2O_2 (positive control without S9 mix) or benzo(α)pyrene (B(α)P, positive control with S9 mix) or H_2O (negative control), were added to the cultures for 3 h at 37 °C. An aliquot of 100 μL of S9 mix was added at the beginning in those cultures with metabolic activation. Cell viability was evaluated immediately after treatment, with a mixture of fluorescein diacetate (FDA) and ethidium bromide (EtBr). FDA permits to detect only living cells (stained green), while EtBr detects only dead cells (stained red) [26]. Two hundred cells were scored for viability in each treatment; and those concentrations inducing more than 70% of viable cells were used to carry out the comet assay [27]. The comet assay was performed as previously described by Singh et al. [28] with minor modifications. Approximately 40,000 cells in 20 μL were carefully resuspended in 75 μL of 0.5% low-melting-point agarose (LMA), layered onto microscope slides pre-coated with 150 μL of 0.5% normal-melting-point agarose (NMA) (dried at 65 °C), and covered with a coverslip and kept at 4 °C until solidification. Then, coverslips were removed and cells were lysed for 2 h at 4 °C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly cold electrophoresis solution.

Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. After electrophoresis, slides were neutralized with two 5 min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, the slides were stained with 60 μL of EtBr (0.4 μg/mL). The images were examined at 400× magnification with a Komet 5.5 image analysis system (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope, equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. Two different experiments were carried out per compound, each experiment with two different replicates. One hundred randomly selected cells (50 from each of the two replicate slides) were analyzed per sample. The per-

centage of DNA in the tail was used to measure DNA damage and data were computed using the Komet version 5.5 software.

2.5. Detection of induced oxidative damage

To determine the level of oxidized bases induced by the treatments, one concentration (10 mM) per each DBP was evaluated. After cell lysis, slides were washed three times (5 min, 4 °C) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0). Then, aliquots of 100 μL of buffer, containing the bacterial enzymes endoIII or FPG (enzyme concentration 1/1,000) or no enzyme (control) were dropped onto the agarose and incubated for 30 min at 37 °C. After enzyme treatments, cell samples were processed as in the standard version of the alkaline comet assay. Two different experiments were performed with two replicates for each. One hundred cells were scored per experiment (50 per replicate).

2.6. Micronucleus test

Three milliliters of TK6 cells (500,000 cells/mL) were set up in complete medium. Aliquots of 30 μL of each DBP concentration were added to the cultures. All treatments were carried out at the beginning of the incubation and cultures were kept for 48 h at 37 °C in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 h after initiation, 50 μL of each DBP concentration was added to 5 mL culture. Mitomycin C (MMC) (0.2 μM for lymphocyte cultures and 0.3 μM for TK6 cell cultures) and distilled water were used as positive and negative controls, respectively. For each DBP concentration and control, two independent cultures were prepared, and for each culture two slides were prepared.

The cytokinesis-blocked micronucleus (CBMN) test was carried out using the standard technique proposed by Fenech [29]. Cytochalasin B (Cyt-B), at a final concentration of 6 μg/mL, was added to each culture (at time 0 and 44 h after PHA stimulation, for the TK6 and lymphocyte cultures, respectively). After incubation, the cells were harvested. The cultures were centrifuged at 150 g for 8 min, and the supernatant was removed. Cells were subjected to hypotonic treatment (5 mL KCl 0.075 M, 4 °C 7 min), and submitted to a new centrifugation. Finally, cells were fixed with methanol/acetic acid (3:1 vol.) at least 3 times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped onto clean microscope slides (two drops of 20 μL each one). After drying, cells were stained with a Giemsa solution for 7 min.

All slides were coded before scoring, which was carried out by the same person using a Leitz-Leica light microscope at 1000× magnification, under oil immersion. The criterion for scoring MN (size, form, staining, etc) was that described by Kirsch-Volders et al. [30]. One thousand binucleated cells were scored and classified, according to the number of MN. In addition, cells were scored to determine the cytokinesis-block proliferation index (CBPI), according to the formula proposed by Surrallés et al. [31]. For each culture, one or more slides were scored for the induction of MN and CBPI and the values presented correspond to the pooled data from the two cultures.

2.7. Statistical analysis

The slide was used as the experimental unit. The percentage of DNA in the tail (% DNA tail) was the parameter assessed in the comet assay. The mean % DNA tail for each slide was determined and the data were compared by means of the one-way analysis of variance (ANOVA). Independent tests using Dunnett's correction for multiple test adjustment were performed to compare each level of concentration to the negative control when the overall *F*-test

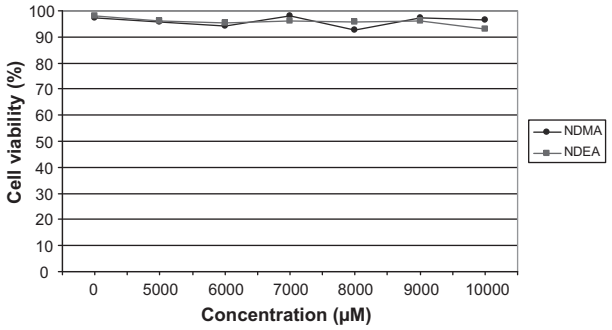

Fig. 1. Viability results after treatment of TK6 cells with NDMA (A) and NDEA (B). Viability was measured by using the FDA/EtBr stain.

was significant. A result was considered statistically significant at $p < 0.05$. For the MN test, data for the binucleated cells with MN were compared for each treatment using the one-tailed Fisher's exact test. The Chi-square test was used for the analysis of CBPI between treatments.

3. Results

According to the FDA/EtBr viability assay (Fig. 1), neither of the nitrosamines was cytotoxic at all, and the highest concentration recommended (10 mM) for *in vitro* experiments did not reduce the cell viability. Consequently, we used concentrations up 10 mM for the genotoxicity assays.

When the genotoxicity of NDMA and NDEA was evaluated in the comet assay, neither compound was effective in inducing primary DNA damage. Thus, the two selected nitrosamines were re-evaluated by using the S9 metabolic fraction and the treatments with or without S9 mix were conducted simultaneously. The results obtained for NDMA (Fig. 2) showed that only the highest concentration tested (10 mM) using S9 mix, was genotoxic. NDEA was slightly more genotoxic since two concentrations (5 and 10 mM) induced significant increases in DNA damage (Fig. 3). These data suggest that the presence of two ethyl groups in the nitrosamine is more genotoxic than the presence of two methyl groups.

The levels of oxidized bases induced by the selected concentration of each nitrosamine (10 mM) were determined by using the treatments with FPG and endoIII enzymes (Fig. 4). Both compounds were unable to induce oxidized pyrimidines; NDMA only induced

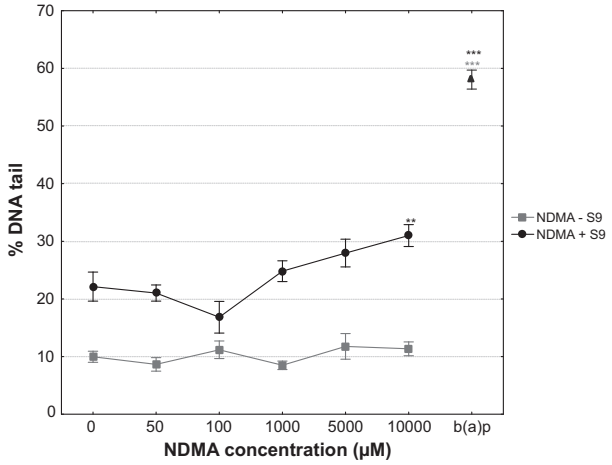

Fig. 2. Genotoxicity of NDMA in the comet assay after 3 h of treatment. Genetic damage is measured as the percentage of DNA in tail. Statistical significance: $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the average of two experiments; bars, SE, H_2O_2 and B(α)P positive controls.

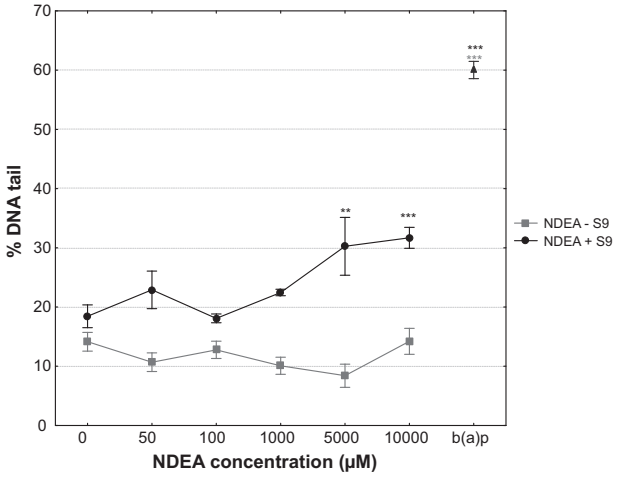

Fig. 3. Genotoxicity of NDEA in the comet assay after 3 h of treatment. Genetic damage is measured as the percentage of DNA in tail. Statistical significance: $^{**}p < 0.01$, $^{***}p < 0.001$. Data represent the average of two experiments; bars, SE, H_2O_2 and B(α)P positive controls.

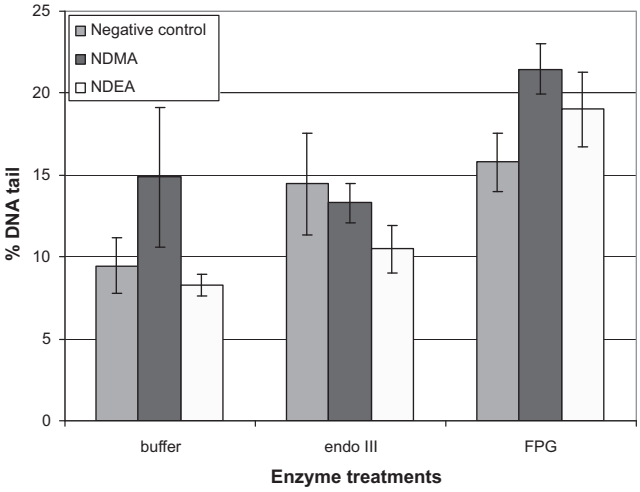

Fig. 4. Effect of the enzyme (endoIII and FPG) treatments in TK6 cells previously treated with 10,000 μM of NDMA and NDEA). Data represent the average of three experiments; bars, SE.

slight damage in purines, whereas the proportion of this kind of damage caused by NDEA was much higher. The oxidative damage induced by NDMA only represents the 5% of the overall damage produced by this chemical, while the oxidative damage produced by NDEA was more than 360% of the overall damage.

The number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei and the CBPI values after treatment with the nitrosamines are summarized in Table 1. The results of the MN test show that both agents were unable to induce significant increases in the frequency of micronuclei. Neither the number of binucleated cells showing at least one MN, nor the frequency of MN scored per 1000 binucleated cells showed any significant increase with respect to the control. Only NDEA was able to produce a decrease in the CBPI values at the highest concentration (10 mM), being more cytotoxic than MMC (positive control).

To be sure that the negative MN results obtained with both nitrosamines were due to their inability to induce fixed genetic damage and not related to the particular characteristics of TK6 cells, an additional experiment was carried out with peripheral blood human lymphocytes to test NDMA (Table 2). As occurs with TK6 cells, none of the evaluated concentrations increases the frequency of MN in binucleated cells. Only increases in the CBPI values, as occurred with the TK6 cells, were observed in the study with lymphocytes. These results confirm the inability of NDMA to induce MN in mammalian cells. In all the MN experiments with TK6 cells and human lymphocytes, the positive control MMC induced clear and significant increases in the frequency of MN.

4. Discussion

The discovery that disinfection procedures generate DBPs in treated water has led to a strong and growing interest in their undesirable health effects, mainly focused on those aspects concerning the potential risk of cancer and adverse reproductive effects. Several epidemiologic studies have shown a possible association between DBP levels and increases in the incidence of cancer of the bladder, colon and rectum [32–40]; but, besides these data from epidemiologic studies, it is necessary to determine whether DBPs act as genotoxicants and exactly what type of genetic damage they produce.

Table 1
Micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with nitrosodimethylamine and nitrosodiethylamine.

Concentration (mM)	Distribution of MN in BN cells					Total MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
NDMA												
0	971	23	2	1	3[a]	42	29	63	222	35	180	2.30
0.5	957	39	3	1	0	48	43	47	175	51	227	2.46
1.0	972	25	3	0	0	31	28	49	218	44	189	2.37
2.5	966	26	7	0	1[b]	45	34	28	179	60	233	2.53
5.0	965	28	4	3	0	45	35	27	191	61	184	2.34
10	976	21	3	0	0	27	24	60	210	46	184	2.34
MMC	891	84	22	3	0	137	109***	95	337	13	55	1.95***
NDEA												
0	976	20	4	0	0	28	24	56	204	37	203	2.37
0.5	980	18	1	1	0	23	20	42	180	40	238	2.47
1.0	986	12	2	0	0	16	14	38	188	44	230	2.47
2.5	977	21	2	0	0	25	23	50	203	42	205	2.39
5.0	982	17	0	0	1	21	18	52	241	39	168	2.31
10	985	13	2	0	0	17	15	187	266	5	42	1.72***
MMC	930	62	6	2	0	80	70**	136	315	13	36	1.83***

***Statistically significant from control ($p \leq 0.001$).
**$p \leq 0.01$.
[a] BN cells with4 MN
[b] BN cells with 5 MN.

Table 2
Induction of micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values in cultured lymphocyte treated with nitrosodimethylamine.

Concentration (μM)	Distribution of MN in BN cells					MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
0	984	16	0	0	0	16	16	316	273	4	7	1.39
0.5	999	1	0	0	0	1	1	248	227	14	11	1.55
1.0	993	6	0	1	0	9	7	232	244	10	14	1.58
2.5	993	7	0	0	0	7	7	243	226	14	17	1.58
5.0	997	2	1	0	0	4	3	238	238	12	12	1.57
10	990	9	1	0	0	11	10	275	195	12	18	1.51
MMC	940	58	2	0	0	62	60***	384	116	0	0	1.23***

Statistically significant from the control (***$p \leq 0.001$).

N-nitrosamines are well-known environmental toxins that can be metabolized into genotoxic agents. Among them, both NDMA and NDEA exert their mutagenic activity after cytochrome P450-dependent α-oxidation; but, depending on which P450 genes are expressed, the mutagenic response may range from 0.0027 to 4.15 revertants/(nmol p-mol) in a bacterial strain expressing CYP3A4 and CYP2A6, respectively [16]. The reactive intermediates of nitrosamine metabolism also have the ability to alkylate nucleophilic sites of DNA producing alkali-labile adducts, which can lead to the formation of abasic sites.

NDMA is the volatile *N*-nitrosamine most commonly encountered in food samples and it is considered as a potent liver, lung and kidney carcinogen. Its genotoxic effects had been studied in both *in vitro* and *in vivo* assays, inducing gene and chromosome mutations, sister-chromatid exchanges, unscheduled DNA synthesis, as well as a weak induction of bone marrow MN in peripheral mammalian reticulocytes [41]. With respect to its ability to induce primary DNA damage, as detected in the comet assay, some studies have reported genotoxic effects for NDMA, but only at high concentrations in human hepatoma cell lines and primary hepatocytes cultures [42–44], which would agree with our data. With respect to the results obtained in the MN test, our findings have shown that NDMA is not able to induce significant increases in the frequency of MN in binucleated cells. These results are different from those reported by Valentin-Severin et al. [43] in HepG2 cells, where they observed significant increases in the number of MN, but at very high concentrations. They are also different from those reported by the U.S. EPA, who classified NDMA as inductor of bone marrow micronuclei *in vivo*. These discrepancies are probably due to the different cell lines used in the various studies. Thus, TK6 cells and peripheral blood lymphocytes have a limited metabolic activity and an external metabolic source (S9) is required for testing non direct mutagens, although this never completely mimics the *in vivo* situation. On the contrary, HepG2 is a hepatic cell line that retains an important metabolic activity.

NDEA results from the chlorination of water containing diethylamine, in the presence of ammonia ions [14]. The EPA has classified the results of NDEA in the mammalian micronucleus test as inconclusive, since the *in vitro* data reveal discordant results or very weak responses. A possible explanation may be the volatility of its mutagenic metabolites [45]. Our results show that only the two higher concentrations (5 and 10 mM) cause significant increases of DNA damage in the comet assay, but only when the S9 mix was added to the cultures. As occurred with NDMA, NDEA was ineffective to increase the frequency of MN in binucleated cells.

To investigate if these nitrosamines are able to produce oxidative DNA damage, we evaluated the effectiveness of NDMA and NDEA in inducing this kind of damage by using endoIII and FPG treatments. Reactive oxygen species may partially contribute to the genotoxic effect of NDMA, as observed in P450 2E1-expressing cells [44,46]. Our results show that only the FPG treatment enhances the DNA damage produced by NDMA and NDEA, indicating that only oxidized purines are induced. Although this effect is slightly enhanced in NDMA treatments, it is markedly increased after NDEA treatments, where the proportion of oxidative damage was even higher than the direct damage produced.

5. Conclusions

Our results indicate that although both selected nitrosamines are weakly genotoxic, according to the results obtained in the comet assay, they are unable to induce clastogenic and/or aneugenic effects, either in TK6 cells or human peripheral blood lymphocytes. Concerning their activity to induce oxidative damage, enzyme treatments revealed that only NDEA was able to produce increased levels of oxidized bases, mainly in purine sites. This would agree with the higher risk attributable to NDEA respect NDMA. Nevertheless, and taking into account the very high concentrations needed to produce DNA damage, our data suggest a low, if existent, genotoxic risk associated with the presence of these compounds in drinking water.

Acknowledgments

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