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Additive Coclastogenicity of Sodium Selenite and Caffeine in CHO Cells Treated with *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine

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ABSTRACT

The clastogenic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in Chinese hamster ovary (CHO) cells and its modulation by Na_2SeO_3 and caffeine were studied by metaphase analysis of chromosome aberrations (CA) as well as by measuring the formation and repair of single-strand (ss) DNA breaks employing hydroxylapatite chromatography. Treatment of CHO cells with MNNG (1.25 or 2.5 × $10^{-5}M$) for 3 h caused CA in 11 and 19% of metaphases scored, respectively. Pretreatment of cells with Na_2SeO_3 (1–5 μg/mL) or caffeine (0.2–2.0 mg/mL) for 2 h resulted in a 2–3.5-fold increase of CA frequency. Addition of both modulators during the mutagen exposure tended to cause a slight inhibition of clastogenic activity of MNNG ($1.25 \times 10^{-5}M$) or had no effect on CA number when MNNG was used at a concentration of $2.5 \times 10^{-5}M$. Posttreatment of CHO cells with Na_2SeO_3 for 20 h after MNNG was ineffective in influencing the number of metaphases with CA, whereas, at these conditions, caffeine enhanced up to 6–7-fold the clastogenic activity of MNNG. Addition of both modulators during the whole experiment, 2 h pretreatment included, resulted in a further significant increase of CA frequency up to the total pulverization of chromosomes in all metaphases scored. The coclastogenic effect of caffeine was greater in this case. The enhancement of chromosome-damaging activity of MNNG by selen-

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ite and caffeine was better expressed when this carcinogen was applied at the higher concentration used. An additive coclastogenic effect was observed in CHO cells treated simultaneously with Na_2SeO_3 and caffeine plus MNNG. In addition, the treatment of CHO cells with MNNG ($5 \times 10^{-6}M$) caused a rapid increase of ssDNA breaks number reaching maximal values after 30–45 min. However, up to 50–60% of MNNG-induced ssDNA breaks were repaired during the first 60–150 min after the mutagen exposure. The 2 h pretreatment of CHO cells with Na_2SeO_3 (2 µg/mL) or the addition of this trace element after MNNG had no effect on formation and repair of MNNG-induced ssDNA breaks. The coclastogenic effect of Na_2SeO_3 in CHO cells treated with MNNG was not directly linked to the induction and disappearance of ssDNA breaks measured by hydroxylapatite chromatography.

Index Entries: MNNG; chromosome aberrations; CHO cells; selenium; caffeine; coclastogenesis.

INTRODUCTION

Selenium is a well known inhibitor of chemical mutagenesis and carcinogenesis (for review, see *1*). Its role as a chemopreventive agent in humans is under study *(2)*. However, it has also been shown, that in some *in vitro* assays and at higher concentrations this trace element causes genetic damage, e.g., DNA fragmentation and an increased UDS in human fibroblasts, mutagenesis in bacteria, and an increased frequency of sister chromatid exchanges and chromosome aberrations in eukaryotic cells, human cells included *(3–7)*. In addition, a comutagenic activity of Na_2SeO_3 was demonstrated in *S. typhimurium* TA1535 grown in a broth supplemented with selenite and then exposed to *N*-methylnitrosourea (MNU) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) *(8,9)*. It should be mentioned, that the effects of selenite on MNU or MNNG mutagenicities varied significantly from potentiation to no effect or inhibition of mutagenesis in dependence on the treatment schedules used, and that a synergistic potentiation of MNU-induced mutagenesis was registered when bacteria cells were pretreated with sodium selenite and caffeine *(9)*, the latter being a well known modulator of chemical mutagenesis *(10,11)*.

A question was raised as to whether sodium selenite could be a comutagen also in eukaryotic cells, and whether its effects will depend on the treatment procedures used as it was shown in bacteria. Indeed, some preliminary data indicated that this trace element could enhance the clastogenic activity of MNNG and tobacco smoke in Chinese hamster ovary (CHO) cells *(12)*.

This paper reports data concerning the effects of Na_2SeO_3 and caffeine on MNNG-induced clastogenicity in CHO cells treated with the

modulators alone or in combination before, during or after the mutagen exposure, respectively.

MATERIALS AND METHODS

Chemicals

MNNG and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium selenite was supplied by Merck (Darmstadt, Germany).

Chromosome Aberration Test

CHO cells were grown in MEM supplemented with 15% fetal calf serum (FCS), streptomycin sulfate (29.6 µg/mL), kanamycin (100 µg/mL), fungizone (2.5 µg/mL), and sodium carbonate (1.0 mg/mL). The stock cultures were maintained in 240-mL plastic culture flasks (Falcon) at 37°C in a water-saturated CO_2 incubator. For each experiment, approx 10^5 CHO cells were seeded on each 22-mm^2 coverslip in 3.5-cm plastic dishes (Falcon) and kept in MEM with 15% FCS at 37°C for 2–3 d. Experiments started when the cells were 60–80% confluent.

Treatment of CHO Cells with MNNG

The carcinogen was dissolved in 1.0 mL of MEM supplemented with 2.5% FCS at concentrations of $1.25 \times 10^{-5}M$ or $2.5 \times 10^{-5}M$ and added for 3 h to the cells. At the end of this incubation (37°C), the coverslips were washed with MEM and incubated further in a fresh MEM supplemented with 15% FCS for another 20 h. During the last 4 h of this post-MNNG incubation period, 0.1 mL of colchicine (0.01% in 2.5 mL MEM) was added to the cell cultures. Some samples were treated additionally with Na_2SeO_3 (1, 2, and 5 µg/mL) or caffeine (0.2, 1.0, and 2.0 mg/mL) added to the medium for 2 h before MNNG, during the MNNG tretament, during the 20 h-incubation of cells after the MNNG treatment, or throughout the experiments starting 2 h before the addition of MNNG. In some cases, the combined effect of both modulators on MNNG-induced clastogenicity was studied by adding concomitantly these two compounds to the cell cultures during the time intervals mentioned above but at concentrations twice lower then those used when the modulators were applied alone.

When the different types of treatments were compleated, the cells were treated with 1.0% sodium citrate solution for 10 min followed immediately by fixation in methanol:acetic acid (3:1) for 10 min. Air-dried slides were stained with 2% orcein in 50% acetic acid:water, dehydrated and mounted. For each sample 100 metaphase plates were analyzed for chromosome aberrations (CA)(breaks and exchanges).

Measurement of Single-Strand (ss) DNA Breaks by Hydroxylapatite Chromatography

CHO cells grown in Eagle's essential medium (Flow Lab.) supplemented with 10% FCS were plated in 72-cm^2 plastic culture flasks (Falcon) at a concentration of 10^6 cells per flask and incubated at 37°C for 24 h. Cells were uniformly labeled for 24 h with ^{3}H-TdR (0.05 μCi/mL, 0.41 Ci/mmol) or ^{14}C-TdR (0.01 μCi/mL, 56 mCi/nmol). Before use the cells were washed free from the radioactive medium and incubated in a fresh medium for 60 min at 37°C. The cells were harvested by trypsinization, resuspended in growth medium (10^5 cells/mL) and then treated with MNNG ($5 \times 10^{-6}M$) for 30 min or 60 min at 37°C. Some samples were treated also with Na_2SeO_3 (2 μg/mL) starting 2 h before MNNG. Cell samples were taken 0, 15, 30, 45, and 60 min after MNNG addition and diluted with fresh medium without FCS at 0°C. An equal number of untreated control cells (^{3}H-TdR- or ^{14}C-TdR-labeled) were added as an internal standard. The MNNG and Na_2SeO_3 were removed by centrifugation of the cells followed by their ressuspension first in a fresh medium and then in PBS (final concentration, 10^6 cells/mL). The samples collected were maintained at 0°C untill lysis.

In order to study the possible influence of Na_2SeO_3 on the repair of ssDNA breaks, some cell samples were further incubated after the MNNG removal at 37°C in a medium containing 10% FCS and Na_2SeO_3 (2 μg/mL). In this case, cell samples were taken for analysis 0, 15, 30, 60, 90, and 150 min after the MNNG treatment.

Cell lysis and unwinding of DNA were carried out according to Rydberg *(13)*. The unwinding solution contained 0.03*M* NaOH, 0.01*M* Na_2HPO_4, 0.9*M* NaCl, pH 12. In 1.0 mL unwinding solution (25°C) 55 μL of cell suspension was added. Care was taken to protect samples from light and to minimize vibration of vials. After 10 min incubation each sample was neutralized with 1.0 mL 0.034*M* HCl (final pH 7.0). These samples were sonicated 10 sec (Ultrasonic W-35 sonifier) and 0.1 mL 8% SDS was then added.

Hydroxylapatite (BioRad, Richmond, CA) chromatography was carried out at 60°C using 0.01*M*, 0.125*M*, 0.25*M*, and 0.5*M* potassium phosphate buffers (pH 6.9). Single- and double-stranded DNA samples were collected in scintilation vials. Radioactivity of each fraction was measured in ACS II scintilation cocktail (Amersham, Arlington Heights, IL). Statistical analysis of data was carried out according to Student's *t*-test.

RESULTS AND DISCUSSION

The data obtained indicated that selenite and caffeine potentiated the clastogenic activity of MNNG in CHO cells. Thus, the 2-h pretreatment of CHO cells with Na_2SeO_3 (1–5 μg/mL) or caffeine (0.2–2.0

mg/mL) increased 2–3.5-fold the percent of metaphases with CA (Table 1). In this case, selenite enhanced up to 2–5-fold the number of breaks and much greater up to 14–16-fold (MNNG, $2.5 \times 10^{-5}M$) the number of exchanges per cell. A similar predominant increase of the exchange-type aberrations frequency was also observed in CHO cells pretreated with caffeine and then exposed to MNNG.

However, added simultaneously with MNNG to the culture medium for 3 h, both selenite and caffeine tended to decrease the number of metaphases with CA. This effect was observed when cells were treated with the lower concentration of MNNG used, which is consistent with a putative, although rather weak one, desmutagenic activity of these two compounds being easely saturated by a larger number of MNNG molecules.

Furthermore, the 20 h-posttretament of CHO cells with caffeine caused an even better expressed, compared to the pretreatment procedure, coclastogenic effect enhancing from 2 to 7 times the number of metaphases with CA. It should be stressed that contrary to the pretreatment procedure, the potentiation of clastogenicity in this case was predominantly owing, to a drastic increase of the number of breaks per cell. At these conditions, selenite had no a significant effect on MNNG-induced chromosome damage in CHO cells.

Addition of Na_2SeO_3 or caffeine throughout the experiments, starting 2 h before the MNNG treatment enhanced further the number of metaphases with CA mainly owing to an increased number of exchanges. The coclastogenic effect of caffeine in this case was better expressed, especially when MNNG was applied at the lower concentration used (Table 1). It seemed likely that the coclastogenic effect of selenite was realized mainly during the 2 h-pretreatment period since when added to the medium throughout the experiments it potentiated the MNNG-induced clastogenicity in an extent comparable to those observed when this trace element was used for pretreatment only. In contrast, the coclastogenic effect of caffeine when added to the medium throughout the experiments seemed to be a summation of its coclastogenic activities, when added before and after the MNNG exposure. It appeared also that mechanisms of caffeine coclastogenic activity in this assay when used for pre- or post-MNNG treatment of CHO cells might be distinct. As mentioned above, the pretreatment of CHO cells with caffeine resulted in an increase mainly of exchange-type aberrations, whereas the post-MNNG treatment enhanced significantly also the number of breaks per cell.

It should be stressed that treatment of cells with caffeine after MNNG exposure and, especially, its addition throughout the experiments caused a severe destruction of chromosomes up to their total pulverization in all metaphases scored (Table 1). A mitotic inhibition was also observed in cells exposed both to selenite or caffeine added after MNNG or throughout the experiments, the caffeine being more effective in this case.

Table 1
Additive Coclastogenic Effect of Sodium Selenite and Caffeine in Chinese Hamster Ovary (CHO) Cells Treated with *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine (MNNG)

Treatment	2 h before MNNG	3 h coincubation with MNNG	20 h after MNNG	Throughout the experiment
MNNG (M), 1.25 x 10–5*M*		11%[a] 0.02[b] 0.07[c]		
M + Na_2SeO_3 (Se), 1 μg/mL	21% 0.10 0.15	9% 0.05 0.05	7% 0.02 0.06	26%[b] 0.17 0.23
M + Se, 2 μg/mL	24%[h] 0.13 0.16	4% 0.01 0.04	10% 0.01 0.12 MI[d]	24%[b] 0.09 0.19 MI
M + Se, 5 μg/mL	19% 0.08 0.13	5% 0.00 0.05	17% 0.08 0.11 MI	16% 0.04 0.18 MI
M + Caffeine (Caf), 0.2 mg/mL	22%[h] 0.17 0.11	13% 0.07 0.08	18% 0.09 0.13 MI	36%[h] 0.25 0.37
M + Caf, 1.0 mg/mL	26%[h] 0.11 0.25	4% 0.01 0.04	59%[h] 0.72 0.24 MI	87%[h] MI, F[e], P[f]
M + Caf, 2.0 mg/mL	35%[h] 0.14 0.33		75%[h] 1.76 0.23 MI	90%[h] MI, F, P
M + Se, 0.5 μg/mL + Caf, 0.1 mg/mL	19% 0.15 0.14	6% 0.02 0.04	12% 0.04 0.09	13% 0.05 0.11
M + Se, 1.0 μg/mL + Caf, 0.5 mg/mL	12% 0.05 0.09	8% 0.01 0.08	22%[h] 0.06 0.17 MI	31%[h] 0.07 0.33 MI
M + Se, 2.5 μg/mL + Caf, 1.0 mg/mL	13% 0.05 0.13	6% 0.02 0.04	48%[h] 0.22 0.33 MI	MI, F, TP[g]
MNNG (M), 2.5 x 10–5*M*		19% 0.17 0.08		
M + Se, 1 μg/mL	68%[h] 0.29 0.95	14% 0.08 0.10	33% 0.22 0.22	73%[h] 0.21 1.65
M + Se, 2 μg/mL	75%[h] 0.29 1.30	20% 0.07 0.17	23% 0.07 0.22	81%[h] 0.23 1.51
M + Se, 5 μg/mL	72%[h] 0.35 1.13	25% 0.12 0.22	25% 0.15 0.15	89%[h] 0.32 1.69
M + Caf, 0.2 mg/mL	60%[h] 0.20 0.92	10% 0.01 0.10	35%[h] 0.27 0.29	91%[h] 0.22 1.86
M + Caf, 1.0 mg/mL	70%[h] 0.29 1.16	20% 0.10 0.14	85%[h] 1.44 1.13 MI, F	MI, F, TP
M + Caf, 2.0 mg/mL	71%[h] 0.19 1.40	30% 0.11 0.27	94%[h] 1.45 0.55 MI, F	MI, F, TP
M + Se, 0.5 μg/mL + Caf, 0.1 mg/mL	63%[h] 0.10 0.98	21% 0.07 0.14	34%[h] 0.13 0.30	68%[h] 0.12 1.37 F, P
M + Se, 1.0 μg/mL + Caf, 0.5 mg/mL	69%[h] 0.19 0.89	22% 0.04 0.26	55%[h] 0.18 0.64 MI	91%[h] 0.65 2.08 MI, F, P
M + Se, 2.5 μg/mL + Caf, 1.0 mg/mL	71%[h] 0.13 1.36	25% 0.07 0.27	70%[h] 0.71 0.69 MI	MI, F, TP

[a]Percent of metaphases with chromosome aberrations (in untreated CHO cells: 0–1%).
[b]Average number of breaks per cell.
[c]Average number of exchanges per cell.
[d]MI, Mitotic inhibition.
[e]F, Fragmentation.
[f]P, Pulverization.
[g]TP, Total pulverization of chromosomes.
[h]$p < 0.05$–$p < 0.001$.

Furthermore, a clear additive coclastogenic effect was established in CHO cells when Na_2SeO_3 and caffeine were added simultaneously to the culture medium before MNNG or throughout the experiments. Thus, the medium supplementation with both modulators at concentrations twice lower then those used when they were applied separately (0.5, 1.0, or 2.5 μg/mL for Na_2SeO_3 and 0.1, 0.5, and 1.0 mg/mL for the caffeine, respectively) caused similar coclastogenic effects as when CHO cells were treated with MNNG plus selenite or caffeine only added at concentrations 1, 2, or 5 μg/mL and 0.2, 1.0, or 2.0 mg/mL, respectively.

It should be stressed that in all cases the coclastogenic effects of these two compounds were better expressed when the higher concentration of MNNG ($2.5 \times 10^{-5}M$) was used implaying a positive correlation with the extent of genetic damage.

As mentioned above, a similar pattern of selenite and caffeine effects on MNU- and MNNG-induced mutagenesis were observed also in *S. typhimurium* TA1535 *(9)*. Pretreatment of bacteria cells with sodium selenite or caffeine caused an enhancement of MNNG mutagenicity, confirming their comutagenic activities, whereas the coincubation of bacteria with these two modulators and MNNG resulted in an inhibition of mutagenesis, thus providing additional evidences for a possible desmutagenic activity of both compounds toward MNNG. However, in contrast to the data obtained in CHO cells, no additive comutagenic effect was observed in *S. typhimurium* TA1535 pretreated concomitantly with Na_2SeO_3 plus caffeine and exposed subsequently to MNNG. Furthermore, the post-MNNG treatment of bacteria with selenite was ineffective in influencing the his^+ revertants rate, whereas caffeine even slightly inhibited the mutagenesis. However, like in bacteria experiments, the effects of selenite on MNNG-induced mutagenesis in CHO cells were also distinct when this trace element was added before or after the mutagen exposure. It seemed likely that the pretreatment of cells with selenite was of some importance for the expression of its comutagenic activity.

The coclastogenic activity of sodium selenite could be linked to impairments of some DNA repair mechanisms. Employing the hydroxylapatite chromatography, an attempt was made to study the formation and repair of single-strand (ss) DNA breaks in CHO cells treated with Na_2SeO_3 (2 μg/mL) and MNNG ($5 \times 10^{-6}M$). The addition of this carcinogen to the culture medium caused a rapid increase of ssDNA breaks number reaching maximal values after 30–45 min. Up to 50–60% of MNNG-induced ssDNA breaks were repaired during the first 60–150 min after the mutagen exposure. However, it appeared that selenite added throughout the experiments starting 2 h before the MNNG treatment had no effect on the number of ssDNA breaks induced by the carcinogen as well as on the intensity of their repair when the trace element was applyed also after the mutagen exposure (data not shown). Taking into account that selenite caused mainly an enhancement of exchange-type aberrations believed to be a result of mispairing *(14)*, a suggestion

could be made that it might influence in some way the formation and/or elimination of mispairs. As mentioned above, the coclastogenic effect of selenite was not directly correlated with the dynamics of ssDNA breaks formation. Previously, it was shown that caffeine also does not inhibit the repair of ssDNA breaks caused by UV light *(15)*.

The well known caffeine-induced potentiation of the chromosome-damaging activity of alkylating agents, MNNG included *(16)* has been ascribed to an inhibition of DNA repair. Caffeine is supposed to inhibit the post-replication repair by suppressing the filling-in of post-replication gaps formed in the newly sinthesized DNA opposite to the damaged bases as well as to inhibit the excision DNA repair, probably, during the last ligation step or to influence on some repair mechanisms operating in G2 *(11,14,16–18)*. In addition, caffeine was also shown to cause a reversion of G2 delay provoked by the extensive DNA damage after treatment of cells with alkylating agents *(19,29)*. The entrance of cells into mitosis with shattered chromosomes results in an increased number of CA up to the total destruction of chromosomes. The data presented revealed a similar picture of severe chromosome damage with expressed fragmentation and pulverization of chromosomes in cases when CHO cells were treated with MNNG plus caffeine at concentrations of 1.0 or 2.0 mg/mL.

A suggestion could be made that the coclastogenic effects of caffeine and selenite observed in the present study could be a result of an interference of these two agents with some, probably not identical, DNA repair mechanisms, which, in some cases may result in synergistic or additive potentiation of the induced genetic damage.

One might conclude that like in bacteria, selenite could be a comutagen also in eukaryotic CHO cells, enhancing in this case mainly the MNNG-induced exchange-type CA, the exact mechanisms of this effect remaining unclear. The coclastogenic activity of selenite was observed after pretreatment, but not after post-MNNG treatment of cells with selenite, whereas caffeine was effecitive in potentiating the MNNG-induced cytogenetic damage both after pre- or posttreatment of cells, probably, operating in these cases via different mechanisms. An additive coclastogenic effect was detected in CHO cells treated simultaneously with selenite and caffeine, thus implying that some similarities between their coclastogenic activities might exist and confirming the neccessity for careful studies of combined effects of modulators of chemical mutagenesis and carcinogenesis.

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