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Genotoxic effects of oestrogens in breast cells detected by the micronucleus assay and the Comet assay

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Cumulative exposure to oestrogen has been linked to increased risk of breast cancer. Whilst oestrogens induce cancers in rodent bioassays it is unclear whether the mechanisms involved are genotoxic and/or epigenetic. The cytokinesis block micronucleus (CBMN) and the alkaline single cell-gel electrophoresis 'Comet' assays were used to examine MCF-7 cells for chromosomal damage and DNA single-strand breaks (SSBs), respectively. The comet-forming activities of oestrogens were also tested in a 72 h primary culture of cells isolated from freshly expressed breast milk. Micronuclei (MN) were scored in 500 binucleate cells per treatment and SSBs were quantified by comet tail length (CTL) (μm). Effects on mitotic rate (per cent binucleate cells) and cell viability (per cent plating efficiency) were also assessed. β-Oestradiol, oestrone and oestriol were tested for genotoxicity in the 10^{-10}–10^{-4} M and 10^{-10}–10^{-2} M concentration ranges in the CBMN and Comet assays, respectively. β-Oestradiol, following 24 h treatment but not 120 h treatment, induced increases (up to 3-fold) in MN at a concentration of 10^{-9} M. Oestrone induced dose-related increases in MN (up to 5-fold) following both 24 and 120 h treatment, whereas oestriol appeared not to induce MN. All three oestrogens induced dose-related increases in per cent binucleate cells suggesting that they enhance mitotic rate. In the Comet assay both β-oestradiol and oestrone induced dose-related increases in SSBs (up to 7-fold over control CTL) and were significantly comet-forming ($P < 0.0001$) at concentrations as low as 10^{-9} and 10^{-8} M, respectively, whereas oestriol was less genotoxic. All three oestrogens were significantly comet-forming ($P < 0.0001$) in a primary culture of breast milk cells, suggesting that they can damage the target cells from which breast cancers may eventually arise.

Introduction

Breast cancer is the most common malignancy occurring in women (Higginson *et al.*, 1992) and rates of mortality from this disease remain high. Less than 5% of breast cancers are associated with a genetic predisposition, with the remaining 95% being classified as sporadic (Higginson *et al.*, 1992). Underlying mechanisms that could explain the aetiology of sporadic breast cancer, most commonly a disease of the post-menopausal years, remain to be elucidated (Martin, 2001; Grover and Martin, 2002). Epidemiological studies of migrant populations implicate environmental exposures as playing a significant role in incidence (Ziegler *et al.*, 1993), but the only environmental exposure proven to cause breast cancer in humans is ionizing radiation (Tokunaga *et al.*, 1987). Numerous agents of dietary and/or environmental origin are proven rodent mammary carcinogens (El-Bayoumy, 1992) and the mutational spectrum in the *TP53* gene in human mammary tumours implicates exogenous agents (Biggs *et al.*, 1993). However, factors that increase oestrogen exposure increase risk for breast cancer whereas those that reduce oestrogen exposure are protective (Feigelson and Henderson, 1996).

The endogenous oestrogens β-oestradiol, oestrone and oestriol are produced in the ovary, placenta, testes and adrenal glands, mainly from cholesterol (Lingeman, 1979), and their production is controlled primarily by pituitary gonadotropins. Physiological concentrations (pg–ng/ml serum concentrations) may fluctuate dramatically depending on menstrual cycle, pregnancy, age or obesity and are essential for the maintenance of cell growth and a multitude of other normal functions (Adashi, 1992). However, oestrogens, including β-oestradiol and oestrone, can induce cancers in different organs in different animal models (Cavalieri *et al.*, 2000). Whilst they have not tested positive in many classical bacterial and mammalian cell gene mutation assays, there is accumulating evidence suggesting that oestrogens may be genotoxins (Roy and Liehr, 1999; Cavalieri *et al.*, 2000). Epigenetic mechanisms may include stimulation of cell proliferation, spontaneous induction of replication errors or disruption of spindle formation and subsequent induction of aneuploidy (Cavalieri *et al.*, 2000; Fischer *et al.*, 2001). However, the exact events that may play a role in oestrogen-induced carcinogenesis remain to be elucidated.

Human mammary epithelial cells (HMECs) possess the enzymes involved in oestrogen biosynthesis (CYP17 and aromatase), the enzymes required for oestrogen hydroxylation (CYP1A1 and CYP1B1) and oestrogen-conjugating enzymes (catechol *O*-methyltransferase) (Williams and Phillips, 2000). Different lifestyle factors, such as alcohol consumption, a risk factor for breast cancer (Bowlin *et al.*, 1997), may increase the levels of circulating oestrogens. In post-menopausal women the primary source of oestrogens is the conversion of androstenedione to oestrone in adipose tissue and, hence, obesity may enhance breast cancer risk (Feigelson and Henderson, 1996). Breast cancer incidence varies markedly between Western countries (high incidence) and countries in the Far East (low incidence) (Parkin *et al.*, 1999; Grover and Martin, 2002). Lower urinary levels of β-oestradiol, oestrone and oestriol are excreted by Singapore Chinese women than US women (Ursin *et al.*, 2001) and this supports a positive association between breast cancer incidence and oestrogen exposure.

Because of the continuing debate over whether oestrogens play a pivotal role in the aetiology of sporadic breast cancer, we have investigated the effects of the three oestrogens, β-oestradiol, oestrone and oestriol, in the MCF-7 cell line originally derived from an oestrogen receptor-positive breast carcinoma and in a primary culture of cells isolated from

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freshly expressed breast milk. The oestrogens were tested for genotoxicity using the cytokinesis block micronucleus (CBMN) and the alkaline single cell-gel electrophoresis 'Comet' assays. Effects on mitotic rate and cell viability were also assessed.

Materials and methods

Chemicals

All chemicals, including test chemicals, were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise stated. Cell culture consumables were obtained from Life Technologies (UK) unless otherwise stated.

Human milk donation

After ethical approval was obtained, a breast feeding mother (of Caucasian ethnic background) was asked to donate samples of freshly expressed milk. A brief questionnaire on dietary, drinking and smoking habits was completed. The donor, labelled BM1, was aged 31 years. This was her first lactation and four separate samples of ~30 ml each were provided on lactation day 5. She consumed meat (red meat, poultry and fish) on a regular basis, consumed three or four portions of fresh fruit per day, was unexposed to cigarette smoke and abstained from alcohol consumption. The samples were immediately transported at 4°C to the laboratory and were processed within 1 h of expression.

Cell culture

The human mammary carcinoma cell line (MCF-7) (a gift from the Institute of Cancer Research, UK) was grown in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. MCF-7 cells were cultured routinely in 75 cm^2 flasks at 5% CO_2 in air and 37°C in a humidified atmosphere and subcultured (1:10 v/v) twice weekly.

Milk samples were diluted 1:1 with RPMI 1640 medium in order to facilitate cell separation (Taylor-Papadimitriou and Stampfer, 1992) and centrifuged at 1000 *g* for 20 min. Cell pellets were washed twice by resuspension in medium and centrifugation. The yield of primary cells was estimated to be 2.44 ± 1.14×10^5/ml breast milk. Cells were then seeded into 12-well multi-well dishes with each well containing 1 ml RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml hydrocortisone (Sigma Chemical Co., UK), 5 μg/ml insulin (Sigma Chemical Co.) and 5 μg/ml cholera toxin (Sigma Chemical Co.) and were incubated at 37°C in 5% CO_2 in air. The medium was changed after 3 days in order to concentrate the proportion of HMECs (Martin *et al.*, 2000).

Prior to subculture or incorporation into experiments, cultured cells were disaggregated, using a 0.05% trypsin/0.02% EDTA solution, to form a single cell suspension.

The CBMN assay

MCF-7 cells were treated for either 24 or 120 h with oestrogens (β-oestradiol, oestrone or oestriol) that were added as solutions in DMSO (maximum concentration 1% v/v) in the CBMN assay. For the 120 h treatment, MCF-7 cells were seeded into 25 cm^2 flasks in the presence of different concentrations, as indicated, of different oestrogens and incubated for 72 h. Following disaggregation with trypsin/EDTA and resuspension in complete medium, 3 ml aliquots (~1×10^4 cells) were seeded into 30 mm Petri dishes containing 20 mm coverslips (Sarstedt, UK). After 48 h the medium was replaced with fresh medium without oestrogen but containing 2 μg/ml cytochalasin B. Cells were cultured for a further 24 h prior to removal of medium, washing of the coverslips with phosphate-buffered saline (PBS) and fixation with 70% ethanol. Cells were then stained with 5% Giemsa prior to mounting of coverslips, in DPX mountant (BDH, UK), on microscope slides.

For the 24 h treatment, routinely cultured MCF-7 cells were disaggregated with trypsin/EDTA, resuspended in complete medium and 3 ml aliquots (~1×10^4 cells) seeded into 30 mm Petri dishes containing 20 mm coverslips. The cells were then allowed to attach for 24 h prior to addition of oestrogens, as indicated. Following 24 h incubation the medium was replaced with fresh medium, without oestrogen but containing 2 μg/ml cytochalasin B and the method above was adopted.

For each treatment condition, micronuclei in 500 binucleate MCF-7 cells from a minimum of three experiments were scored. Mitotic rate was assessed as per cent binucleate cells (mean ± SD, $n = 3$).

The Comet assay

Alkaline lysis followed by alkaline gel electrophoresis was employed to detect DNA single-strand breaks (SSBs) (Martin *et al.*, 1997, 1999, 2000). MCF-7 or cultured breast milk cells were incubated at 37°C for 2 h in the presence or absence of oestrogens (β-oestradiol, oestrone or oestriol) that were added as solutions in DMSO (maximum concentration 1% v/v) with or without the DNA repair inhibitors hydroxyurea (HU) (1 mM) and cytosine arabinoside (ara-C) (120 μM). Following trypsin/EDTA disaggregation, cells were resuspended in PBS and mixed 1:1 with 1% low melting point agarose (Life Technologies, UK). Aliquots (120 μl) were then applied to microscope slides pre-coated with 1% normal melting point agarose (Life Technologies, UK) and evenly distributed following the application of coverslips. The slides were then placed on a cold surface for 5 min. Coverslips were carefully removed and the slides submerged in cold lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO), protected from light and stored at 4°C for at least 1 h. Slides were then transferred to a horizontal electrophoresis tank, covered in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13, freshly prepared) and stored for 20 min to allow DNA unwinding prior to electrophoresis at 0.8 V/cm and 300 mA for 24 min. After electrophoresis, slides were neutralized (0.5 M Tris, pH 7.5) and stained with ethidium bromide (20 ng/ml), after which comet tail length (CTL) (μm) was visualized by epifluorescence using a Leitz Dialux 20 EB microscope. A total of 50 digitized images/data point, 25 from each of two duplicate slides, was measured in each experiment. Experiments were repeated independently at least five times and the results from representative experiments are shown. CTL measurements obtained from treated cell populations were compared to corresponding controls using a Mann–Whitney test.

The clonogenic assay

Following disaggregation of routinely cultured MCF-7 cells with trypsin/EDTA and resuspension in complete medium, 5 ml aliquots containing 1×10^3 cells were seeded into 25 cm^2 flasks in the presence or absence of graded concentrations of oestrogens, as indicated. Oestrogens were added as solutions in DMSO (maximum concentration 1% v/v). The cells were incubated in 5% CO_2 in air at 37°C in a humidified atmosphere for 24 h. The medium was then replaced with fresh medium without oestrogen. Cells were cultured undisturbed for a further 7 days prior to removal of medium, washing with PBS and fixation with 70% ethanol. Colonies were then stained with 5% Giemsa, counted and plating efficiencies calculated.

Results

The effects of β-oestradiol, oestrone and oestriol in the CBMN assay following 24 or 120 h treatment of MCF-7 cells are shown in Figures 1 and 2, respectively. Marked differences in activity dependent on concentration and length of treatment time were observed with different oestrogens. Micronuclei were scored in populations of 500 binucleate MCF-7 cells either as micronucleated binucleate cells, total number of micronuclei or the distribution of micronuclei in binucleate cells.

Following 24 h treatment, 10^{-9} M (1 nM) β-oestradiol induced a 3-fold increase in micronucleus-forming activity (Figure 1). Higher concentrations (10^{-7}, 10^{-5} or 10^{-4} M) induced dose-related decreases in micronucleus formation. Similar increases in micronucleus formation were observed at concentrations as low as 10^{-10} M, but these increases did not appear to be dose related (data not shown). The induction of micronucleus formation by β-oestradiol was characterized by increases in single micronuclei in binucleate cells. Following 120 h treatment dose-related decreases in β-oestradiol-induced micronucleus-forming activity were observed (Figure 2).

Oestrone (10^{-10}, 10^{-8}, 10^{-6} or 10^{-4} M) induced dose-related increases in micronucleus-forming activity following both 24 and 120 h treatment (Figures 1 and 2). Up to 5- and 2-fold increases in micronucleus formation were observed following 24 and 120 h treatment, respectively. A feature of oestrone-induced micronucleus-forming activity was the dose-related induction of multiple micronuclei in binucleate MCF-7 cells. In contrast to these observations, oestriol (10^{-10}, 10^{-8}, 10^{-6} or 10^{-4} M) was observed to induce dose-related decreases in micronucleus-forming activity in comparison with background control levels following both 24 and 120 h treatment (Figures 1 and 2).

Figure 3 shows the effect of 24 or 120 h treatment with

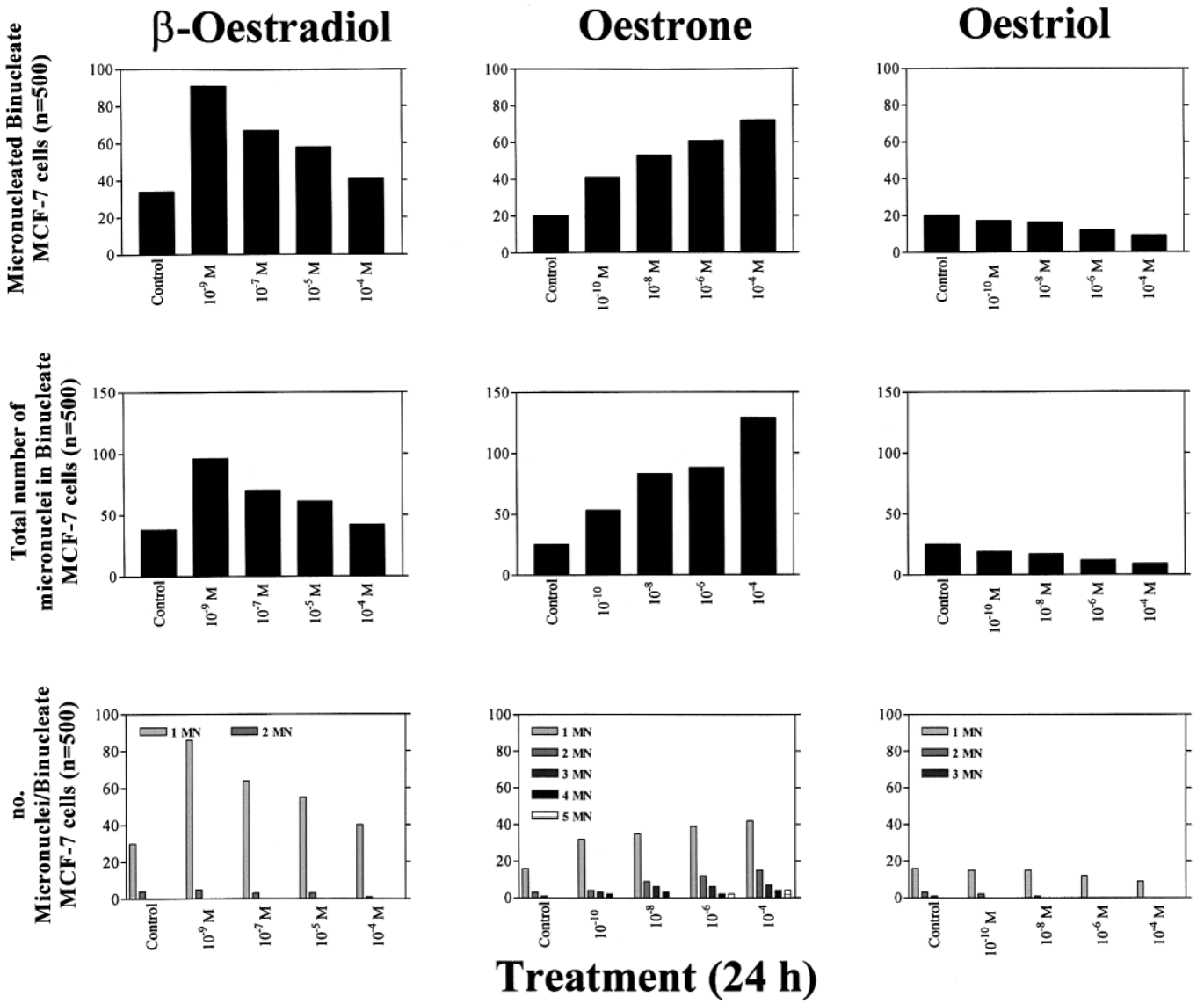

Fig. 1. Micronucleus-forming activity of β-oestradiol, oestrone and oestriol following 24 h treatment in MCF-7 cells. Cells were seeded as 3 ml aliquots (~1×10^4 cells) into 30 mm Petri dishes as described in Materials and methods. Following treatment the cells were blocked at cytokinesis by addition of fresh medium containing 2 μg/ml cytochalasin B. Cells were cultured for a further 24 h prior to fixation and staining with 5% Giemsa. Micronucleus formation was scored in 500 binucleate cells.

β-oestradiol, oestrone or oestriol on per cent binucleate MCF-7 cells following cytokinesis block. Following 24 h treatment, all three oestrogens induced dose-related increases (up to 2-fold) in per cent binucleate cells. However, following 120 h treatment with β-oestradiol or oestriol, dose-related increases (up to 4-fold) in per cent binucleate cells were observed, whereas 120 h treatment with oestrone appeared not to induce any increase.

Figures 4 and 5 show the data obtained when the comet-forming effects of β-oestradiol, oestrone or oestriol in MCF-7 cells in the presence or absence of the DNA repair inhibitors (HU/ara-C). In the absence of HU/ara-C, 10^{-9} M β-oestradiol induced increases in CTL (median 31.69 μm, $P < 0.002$) as opposed to control CTL (median 21.57 μm). However, in the presence of HU/ara-C, 10^{-9} M β-oestradiol induced increases in CTL (median 68.26 μm, $P < 0.0001$) as opposed to control CTL (median 22.74 μm). In 10^{-9} M β-oestradiol-treated MCF-7 cells a significantly higher ($P < 0.0001$) CTL was observed in the presence than in the absence of HU/ara-C. These effects were even more pronounced following treatment with 10^{-7} M β-oestradiol. Figure 5 shows that in the presence of HU/ara-C nanomolar concentrations of both β-oestradiol and oestrone induce significant increases ($P < 0.0001$) in comet-forming activity, whereas oestriol becomes active at 10^{-8} M ($P < 0.001$) and 10^{-6} M (1 μM) ($P < 0.0001$).

β-Oestradiol, oestrone and oestriol all increased plating efficiency at the lower concentrations tested [10^{-9} and 10^{-7} M β-oestradiol ($P < 0.01$); 10^{-10} and 10^{-8} M oestrone ($P < 0.001$) and oestriol ($P < 0.01$)] (Figure 6). Significant reductions in plating efficiency were observed at higher concentrations of β-oestradiol [10^{-5} and 10^{-4} M ($P < 0.01$ and $P < 0.0001$, respectively)] and oestrone [10^{-4} M ($P < 0.05$)]. Oestriol did not appear to reduce plating efficiency at concentrations up to 10^{-4} M (Figure 6).

In a 72 h primary culture of breast milk cells all three oestrogens were found to induce significant comet-forming activities ($P < 0.0001$) only in the presence of the DNA repair

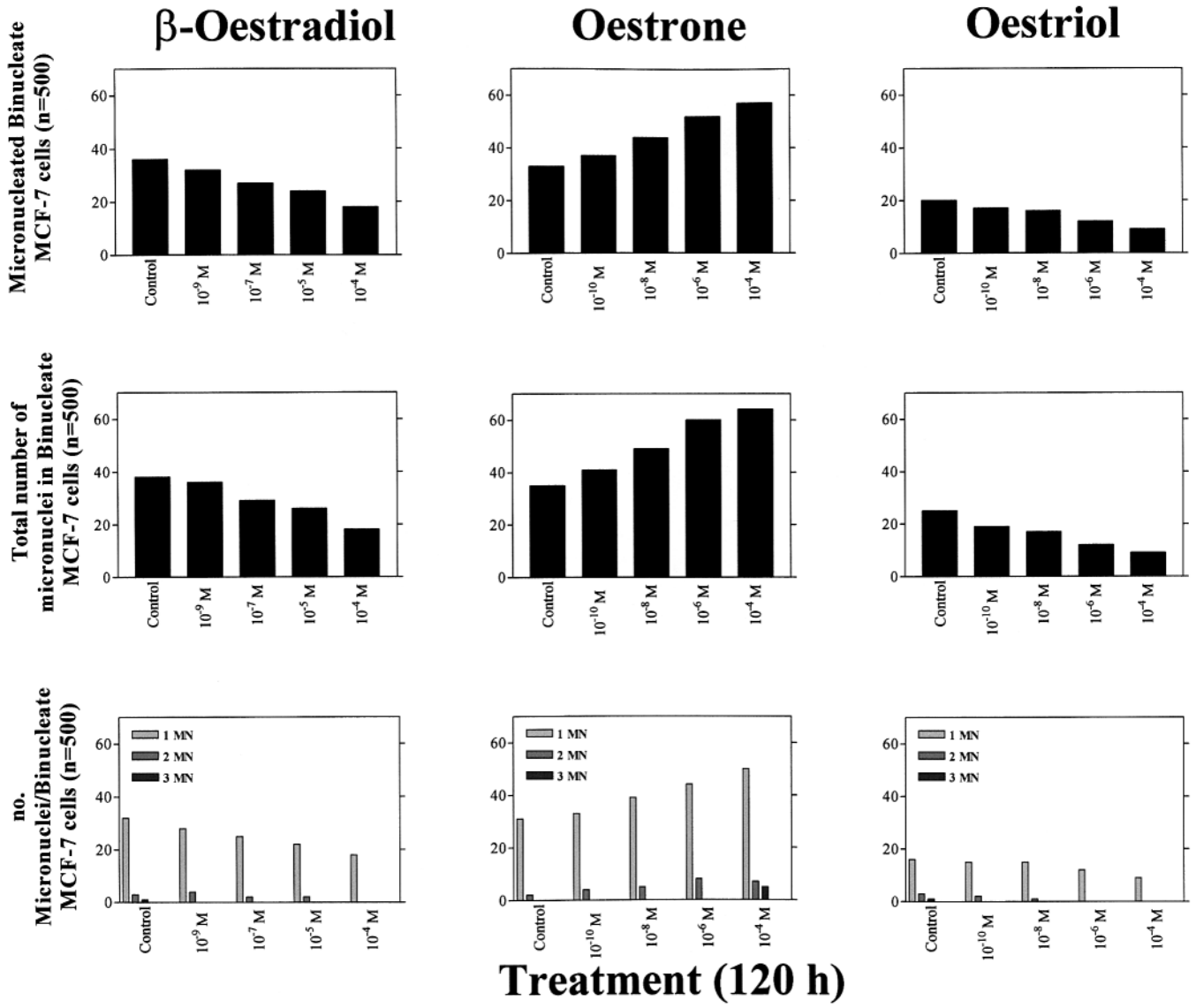

Fig. 2. Micronucleus-forming activity of β-oestradiol, oestrone and oestriol following 120 h treatment. Cells, pre-treated for 72 h with different oestrogens as indicated, were seeded as 3 ml aliquots (~1×10^4 cells) into 30 mm Petri dishes as described in Materials and methods. Following treatment for a further 48 h, the cells were blocked at cytokinesis by addition of fresh medium containing 2 μg/ml cytochalasin B. Cells were cultured for a further 24 h prior to fixation and staining. Micronucleus formation was scored in 500 binucleate MCF-7 cells.

inhibitors HU/ara-C (Figure 7). β-Oestradiol (10^{-9} M), oestrone (10^{-8} M) or oestriol (10^{-8} M) in the presence of HU/ara-C induced median CTLs of 95.56 ($P < 0.0001$), 78.08 ($P < 0.0001$) and 63.99 μm ($P < 0.0003$), respectively, compared to a background level of 25.60 μm. In the absence of HU/ara-C oestrogen-induced comet formation was not observed in cultured breast milk cells from this particular donor.

Discussion

The notion that oestrogens, hormones required for physiological processes, may play a role in the aetiology of breast cancer remains controversial. Oestrogens are necessary for cell turnover and proliferation and normal functioning of several organs other than the breast (Roy and Liehr, 1999). Commencing from the observations by Ramazzini of a higher incidence amongst nuns (Ramazzini, 1940, originally published 1713) up to the most recent reports that cumulative oestrogen exposure enhances risk (Feigelson and Henderson, 1996), there is a large body of circumstantial evidence supporting a role for oestrogens in breast cancer aetiology. The present study has investigated the effects of oestrogens, in the CBMN and Comet assays, using the oestrogen receptor-positive breast carcinoma MCF-7 cell line and a primary culture of breast milk cells.

Dose-dependent effects of oestrogen on micronucleus formation were observed (Figures 1 and 2). At the lower concentrations tested β-oestradiol, following 24 h treatment, induced a marked increase in binucleate cells possessing single micronuclei. Whether these observations result from an induction of numerical changes such as aneuploidy brought about by β-oestradiol-induced proliferation remains to be investigated. Significant increases ($P < 0.0005$) in per cent binucleate cells, as a measure of β-oestradiol-induced proliferation, were seen following both 24 and 120 h treatment (Figure 3). However, an inverse relationship between micronucleus formation and proliferation was observed following

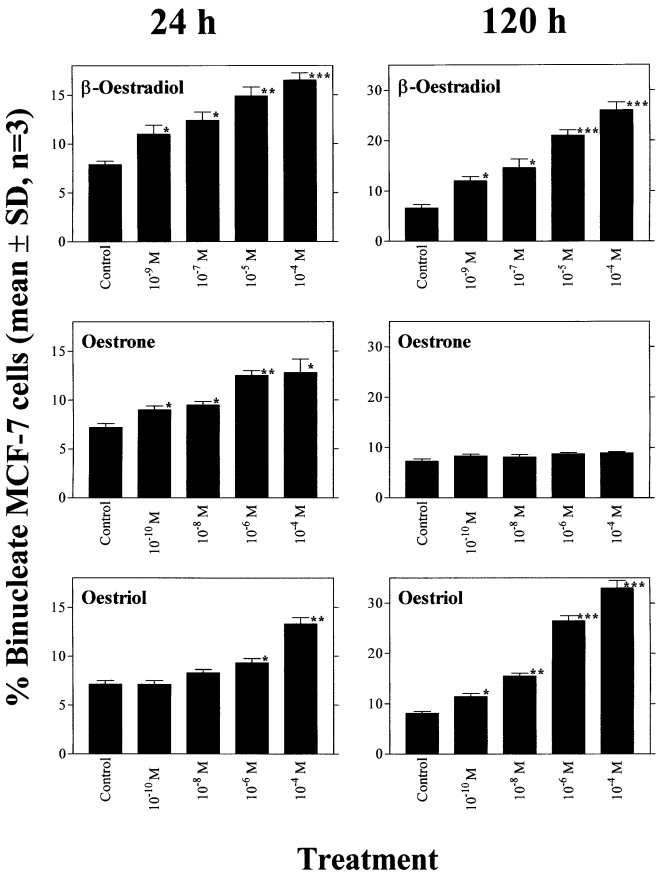

Fig. 3. Effects of β-oestradiol, oestrone and oestriol on mitotic rate following 24 and 120 h treatment of MCF-7 cells. After treatment, cells were blocked at cytokinesis by addition of fresh medium containing 2 μg/ml cytochalasin B as described in Materials and methods. Cells were cultured for a further 24 h prior to fixation and staining. Mitotic rate was estimated as per cent binucleate MCF-7 cells from the mean ± SD of three separate counts. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$ (treatment versus control) as determined by Student's *t*-test.

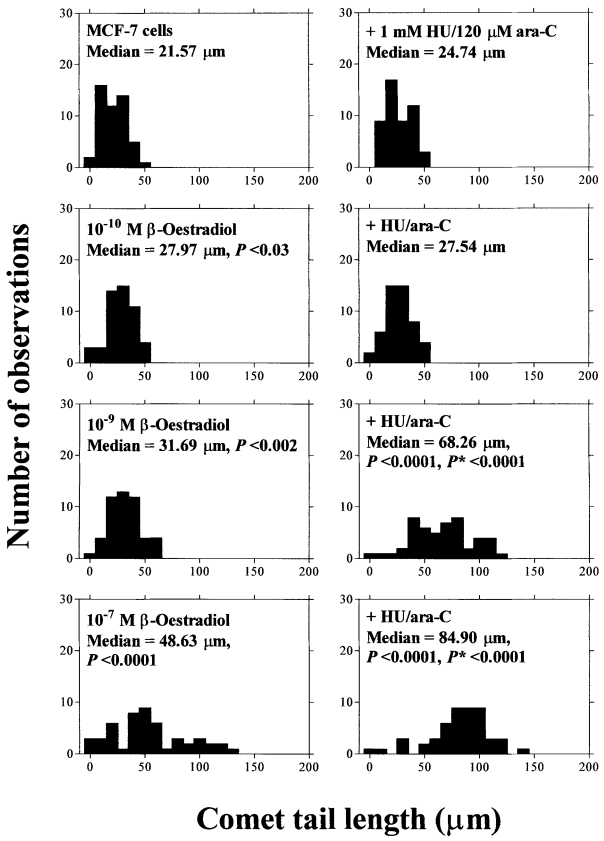

Fig. 4. Comet-forming activity of β-oestradiol in MCF-7 cells. Cells were grown to confluence prior to treatment with β-oestradiol in the presence or absence of the DNA repair inhibtors hydroxyurea and cytosine arabinoside (HU/ara-C, 1 mM and 120 μM final concentrations). Following a 2 h treatment, cells were disaggregated with trypsin/EDTA prior to incorporation into the Comet assay as described in Materials and methods. Comet tail length (μm) was used as a measure of DNA damage. *P*, as compared with corresponding control; *P**, as compared with the corresponding treatment in the presence of HU/ara-C.

24 h treatment with higher concentrations of β-oestradiol and with all concentrations tested following 120 h treatment with β-oestradiol (Figures 1–3). Whether this was an effect of enhanced proliferation diluting the proportion of micronucleated binucleate cells or was due to oestrogen-induced cell death also remains to be elucidated.

The effects of oestrone on micronucleus formation, as shown in Figures 1 and 2, are more typical of the pattern of micronucleus formation induced by a classic genotoxic agent e.g. benzo[*a*]pyrene (C.Davis, S.Bhana, A.J.Sharrocks and F.L.Martin, manuscript in preparation). Dose-related increases in micronucleated binucleate MCF-7 cells were observed with increasing proportions of binucleated cells possessing multiple micronuclei and resulting in a greater number of total micronuclei (Figure 1). After DNA adduct formation with reactive metabolites of oestrone (Cavalieri *et al.*, 2000), it is possible that oestrone-induced micronuclei result from clastogenic effects and, as a product of repair-induced strand breaks, represent acentric fragments. Oestrone-induced proliferation seen following 24 h treatment appears to be inhibited following 120 h treatment (Figure 3) and this may suggest a cell cycle arrest. However, whether oestrone-induced effects are indicative of genotoxic effects requires further investigation.

Whilst oestriol induced pronounced dose-related increases in per cent binucleate cells (Figure 3) following both 24 and 120 h treatment, it appears to induce dose-related decreases in micronucleus formation following both treatments (Figures 1 and 2). This result points to a dissociation between oestrogen-induced micronucleus formation and proliferation. Whether oestriol inhibits micronucleus induction through regulation of apoptosis or other as yet unidentified mechanisms is unknown. However, these data support observations that oestriol appears to be less carcinogenic than β-oestradiol or oestrone and was, in some cases, protective in rodent bioassays (Lemon *et al.*, 1989).

In MCF-7 cells both β-oestradiol and oestrone induced clear dose-related increases in CTL in the Comet assay whereas oestriol appeared to be less comet-forming (Figures 4 and 5). The sensitivity of the Comet assay can be enhanced by the inclusion of DNA repair inhibitors (HU/ara-C) (Martin *et al.*, 1999; Pfau *et al.*, 1999). Significant increases ($P < 0.0001$) in CTL over control levels were observed following treatment of MCF-7 cells with 1 nM β-oestradiol in the presence of HU/ara-C (Figure 4). Production rates of 100–300 and 100–200 μg/day for β-oestradiol and oestrone, respectively, are observed in non-pregnant women and during pregnancy milligram amounts are secreted (Adashi, 1992). Taken together, these results imply that oestrogens are capable of inducing DNA damage in breast cells at physiological concentrations.

Oestrogen-induced genotoxic mechanisms may include

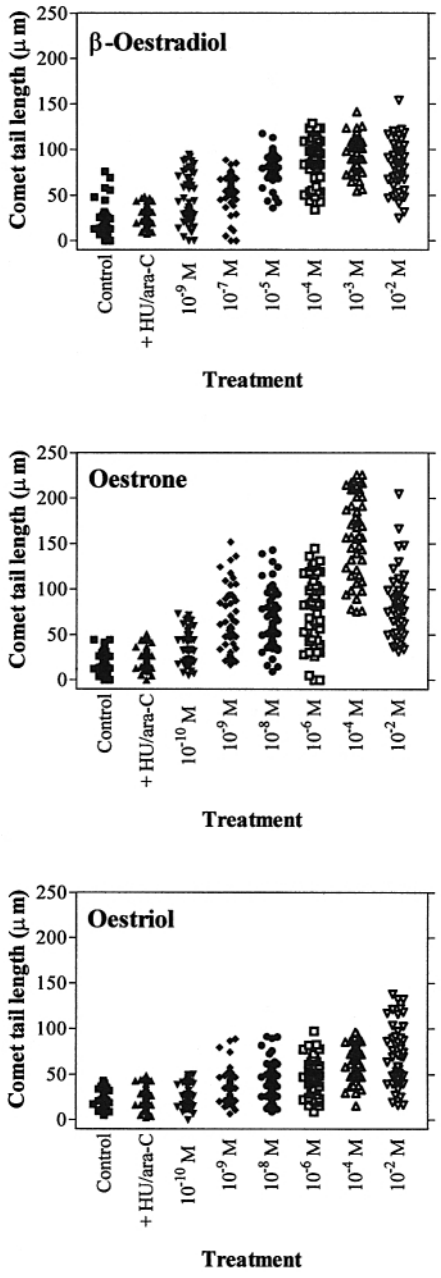

Fig. 5. Comet-forming activity of β-oestradiol, oestrone and oestriol in MCF-7 cells. Cells were grown to confluence prior to treatment with oestrogens in the presence of the DNA repair inhibitors hydroxyurea and cytosine arabinoside (HU/ara-C, 1 mM and 120 μM final concentrations). Control cell populations in the presence of the vehicle (DMSO) were incubated in the presence or absence of HU/ara-C. Following a 2 h treatment, cells were disaggregated with trypsin/EDTA prior to incorporation into the Comet assay as described in Materials and methods. Comet tail length (μm) was used as a measure of DNA damage. Median CTLs (significance) were as follows. β-Oestradiol: control, 19.62 μm; +HU/ara-C, 24.74 μm; 10^{-9} M, 39.25 μm ($P < 0.002$); 10^{-7} M, 57.17 μm ($P < 0.0001$); 10^{-5} M, 86.18 μm ($P < 0.0001$); 10^{-4} M, 90.19 μm ($P < 0.0001$); 10^{-3} M, 104.1 μm ($P < 0.0001$); 10^{-2} M, 84.47 μm ($P < 0.0001$). Oestrone: control, 18.94 μm; +HU/ara-C, 22.15 μm; 10^{-10} M, 32.87 μm ($P < 0.003$); 10^{-9} M, 58.45 μm ($P < 0.0001$); 10^{-8} M, 59.30 μm ($P < 0.0001$); 10^{-6} M, 83.62 μm ($P < 0.0001$); 10^{-4} M, 168.80 μm ($P < 0.0001$); 10^{-2} M, 78.93 μm ($P < 0.0001$). Oestriol: control, 21.62 μm; +HU/ara-C, 22.95 μm; 10^{-10} M, 26.00 μm; 10^{-9} M, 35.74 μm ($P < 0.003$); 10^{-8} M, 43.58 μm ($P < 0.001$); 10^{-6} M, 49.29 μm ($P < 0.0001$); 10^{-4} M, 66.76 μm ($P < 0.0001$); 10^{-2} M, 66.66 μm ($P < 0.0001$).

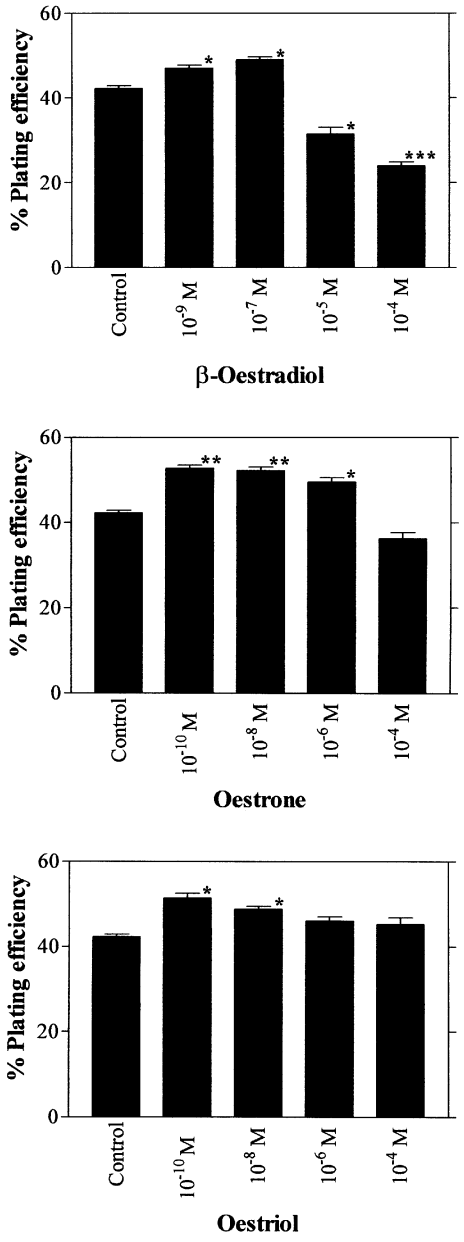

Fig. 6. Effects of β-oestradiol, oestrone and oestriol on per cent plating efficiency in MCF-7 cells. Cells (1×10^3) were seeded in 25 cm^2 flasks in the presence or absence of oestrogens and incubated for 24 h, as described in Materials and methods. Following addition of fresh medium, in the absence of oestrogen, cells were cultured undisturbed at 37°C and 5% CO_2 in a humidified atmosphere for 7 days. Surviving colonies were fixed and stained and per cent plating efficiency was calculated by estimating the percentage of colonies counted over the number of cells initially seeded. $^*P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$ (treatment versus control) as determined by Student's *t*-test.

DNA adduct formation by oestrogen metabolites, free radical generation by redox cycling of oestrogens through microsomal, mitochondrial or nuclear processes or oxidative stress catalysed by the presence of Cu^+ or Fe^{2+} (Roy and Liehr, 1999; Cavalieri *et al.*, 2000). Despite the DNA-damaging effects of β-oestradiol and oestrone, they and oestriol induced increases in plating efficiency at the concentrations employed in both the CBMN and Comet assays (Figure 6). β-Oestradiol is also known to interfere with the control of apoptosis (Leung and Wang, 1999). Genotoxic effects in cells that survive and proliferate are potentially important for the carcinogenic process.

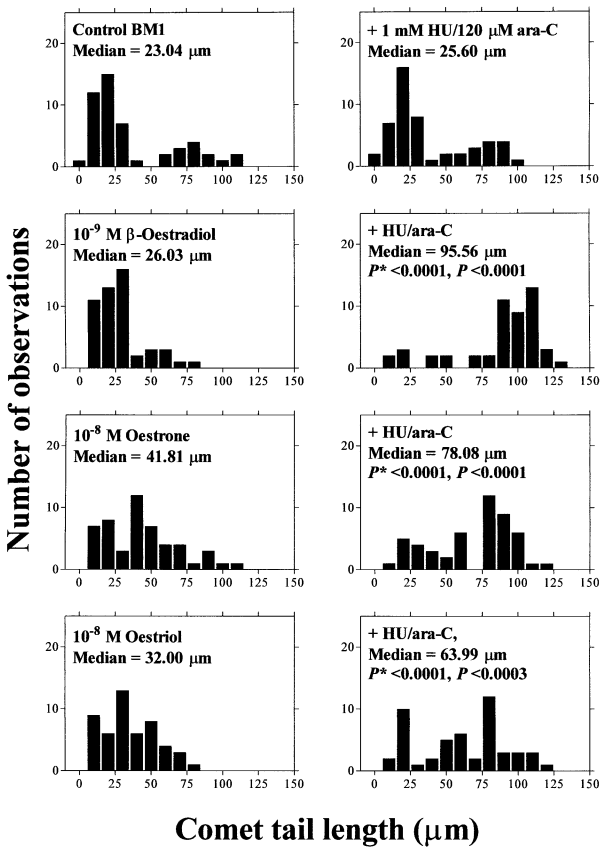

Fig. 7. Comet-forming activity of β-oestradiol, oestrone and oestriol in a primary culture of breast milk cells. Following isolation from freshly expressed breast milk, the cells were cultured for 72 h prior to addition of fresh medium. Breast milk cells were then treated with either 10^{-9} M β-oestradiol, 10^{-8} M oestrone or 10^{-8} M oestriol in the presence or absence of the DNA repair inhibtors hydroxyurea and cytosine arabinoside (HU/ara-C, 1 mM and 120 μM final concentrations). Control cell populations in the presence of the vehicle (DMSO) were incubated in the presence or absence of HU/ara-C. Following a 2 h incubation, cells were disaggregated with trypsin/EDTA prior to incorporation into the Comet assay as described in Materials and methods. Comet tail length (μm) was used as a measure of DNA damage.

Significantly, all three oestrogens induced marked increases in SSBs, in the presence of repair inhibitors, in a primary culture of breast milk cells (Figure 7). Whilst this experiment needs to be repeated using milk samples donated by a larger cohort of individuals, we believe that this is the first demonstration of damage produced by oestrogens in cells from which breast cancers may eventually arise. Genetic polymorphisms and varying lifestyle factors may mean that HMECs from different individuals may well differ in susceptibility to the DNA-damaging effects of oestrogens and further investigations in this area will be necessary.

The effects observed in this study may have implications for the aetiology of breast cancer. Global variations in incidence (Higginson *et al.*, 1992) implicate environmental factors and these factors may be responsible for the differences in oestrogen levels (Ursin *et al.*, 2001) and genotoxin levels (Martin *et al.*, 2001) measured in biological samples obtained from different populations. The potential for oestrogens and classic carcinogens to interact synergistically at concentrations relevant to levels of human exposure also remains to be investigated.

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