Effects of Benzopyrene-7,8-Diol-9,10-Epoxide (BPDE) In Vitro and of Maternal Smoking In Vivo on Micronuclei Frequencies in Fetal Cord Blood

Marta Zalacaín[1], Luis Sierrasesúmaga[1], Carlos Larrañaga[2], and Ana Patiño-García[1]

[1] Department of Pediatrics, University Clinic and University of Navarra, 31080 Pamplona, Spain;
[2] Department of Gynecology and Obstetrics, Virgen del Camino Hospital, 31008 Pamplona, Spain

ABSTRACT

Up to 20% of pregnant women smoke and there is indirect evidence that certain tobacco-specific metabolites can cross the placental barrier and are genotoxic to the fetus. The presence of micronuclei results from chromosome damage and reflects the degree of underlying genetic instability. Fetal blood was obtained from the cord blood of 143 newborns (102 from nonsmoking mothers and 41 from mothers smoking >10 cigarettes/d during pregnancy). The micronucleus assay was performed following the guidelines established by the Human MicroNucleus project with modifications. To test the micronucleus assay, we evaluated the effect of a range of benzopyrene-7,8-diol-9,10-epoxide concentrations (from 3.125 nM to 4 µM on cord blood from nonsmoking mothers. This validation showed that the number of micronuclei and apoptotic cells increased with benzopyrene-7,8-diol-9,10-epoxide dose ($p < 0.0001$ and $p = 0.001$, respectively); the minimal detectable effect was induced by 12.5 nM benzopyrene-7,8-diol-9,10-epoxide. In our sample, the number of MN was significantly higher in the 41 cord blood samples from mothers who smoked during pregnancy [smokers: 4 (1; 10.5); non-smokers: 3 (0; 8); $p = 0.016$]. Therefore, the data reported herein support the hypothesis that tobacco compounds are able to induce chromosomal losses and breaks that are detectable as an increased number of micronuclei.

Correspondence: Ana Patiño García, Ph.D., Laboratory of Pediatrics, Los Castaños Building, University of Navarra, 31080 Pamplona, Spain; e-mail: apatigar@unav.es

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ABBREVIATIONS

BPDE, benzopyrene-7,8-diol-9,10-epoxide; cyt-B, cytochalasin-B; HUMN, Human MicroNucleus project; MN, micronucleus/micronuclei; THF, tetrahydrofuran

INTRODUCTION

Tobacco carcinogenicity is dependent on the activation of solid phase neutral compounds in the smoke by organic enzymes (CYP1A1, GST). Once activated, these compounds are able to induce molecular alterations (mutation in target genes) and cellular alterations (chromosomal aberrations and micronuclei induction). The activated molecule capable of causing most genetic damage is BPDE, which is able to establish covalent bonds with DNA and derives from benzopyrene (BP, neutral-solid phase) after a series of biotransformations mediated by the P450 enzymatic system (1,2).

Tobacco consumption among women is an important problem, given the increasing rate and effects on fertility and gestation (3); it is estimated that 12–20% of women smoke during pregnancy (4), although the exact rate is difficult to establish because many pregnant women deny being smokers in their first clinical consultation. Certain tobacco compounds can cross the placental barrier and induce alterations in fetal development and growth (5). The presence of tobacco-specific metabolites has been detected in the fetal fluids and urine of fetuses and newborns, which strongly suggests a genotoxic effect of tobacco smoke upon newborns from mothers who smoke during pregnancy (6,7). Although different studies have demonstrated the presence of chromosomal aberrations, sister chromatid exchanges and/or increased number of micronuclei in peripheral blood from smokers, which reflect different types of genetic instability, the effect of the genotoxic agents on the fetus is poorly documented. Recently, de la Chica and coworkers (8) have demonstrated that smoking 10 cigarettes or more during pregnancy significantly increases chromosomal instability in amniocytes represented by an increased number of gaps, breaks, and structural chromosomal aberrations. What is more, they detected that the chromosomal region most affected by tobacco smoke was 11q23, a region frequently altered in different neoplasias of hematologic origin.

MN are cytoplasmic bodies of nuclear origin corresponding to genetic material such as whole chromosomes or chromosome fragments, which, during cell division, have not been incorporated in the main nuclei of daughter cells. The unincorporated chromosomal material is the result of chromosome breaks and/or errors during the replication and division of DNA and reflect the underlying genetic instability (9). The use of MN as a method to estimate genetic instability was improved with the development of the cytokinesis-block micronucleus (CBMN) assay, which allows MN to be scored specifically in cells that have completed only one nuclear division. The CBMN assay has since been validated by an international and multicentric committee (HUMN) (10) and is one of the most commonly used methods for evaluating the effects of potential mutagens on human lymphocytes.

Some of the studies on MN frequency in human populations found that cigarette smoke did not affect MN frequency (11–13), whereas many others (14–16) found that smoking and increased MN index were positively correlated. These different results may be due

to differences in definitions of the eligibility criteria of the smoking group. Two recent publications (17,18) detected a significant increase in MN frequency in individuals smoking more than 20 cigarettes per day.

The aims of the study reported here were to establish and validate a MN assay protocol applicable to cord blood samples and then to use this assay to assess the genotoxic effect of tobacco derivatives on the fetal blood of newborns whose mothers smoked during pregnancy.

METHODS

Patients and samples

Fetal blood samples with CPDA-1 as anticoagulant (CPDA, citrate, sodium phosphate, dextrose and adenine; Terumo Medical Corporation, Somerset, NJ) were obtained from the cord blood of 143 newborns (102 from mothers who never smoked and 41 from mothers who were smoking more than 10 cigarettes per day during pregnancy). The study was approved by the Human Ethics Committee of the University Clinic and Virgen del Camino Hospital in Pamplona, Spain. All participants were informed about the protocol and written informed consent was obtained.

Only samples from pregnancies without complications and that concluded in spontaneous births at term (37–42 wk) were included. The samples came from a randomly chosen consecutive series of women who fulfilled the inclusion criteria described above and who were matched for age. In all cases, both the mother and father completed a questionnaire covering their medical history, exposure to chemicals, and passive and active tobacco smoke exposure at home and at work. For a subset of randomly selected cases and for all in which mother and father gave contradictory answers in their questionnaires (n = 10), the cotinine levels, the main metabolite of nicotine, were determined in maternal urine by gas chromatography-mass spectrometry, which has a sensitivity of 20 ng/mL (Reference Laboratory, Barcelona, Spain) (19).

MN assay

Fresh blood was diluted 1:1 with Hank's solution (Invitrogen, Carlsbad, CA) and lymphocytes were isolated by red blood cells sedimentation by Lymphoprep gradient (Nycomed, Roskilde, Denmark). Cell concentration was measured using a hemocytometer and 500,000 viable cells (determined by trypan blue staining) were cultured in RPMI 1640 medium (Invitrogen) containing 10% FCS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Lymphocytes were then stimulated by adding phytohemagglutinin (PHA-P, Sigma Chemical Co., St. Louis, MO) and incubated at 37°C/5% CO_2. Forty-four hours after PHA stimulation cyt-B (Sigma Chemical Co.) was added to block cytokinesis. Twenty-eight hours after cyt-B addition, cells were harvested by centrifugation, hypotonically treated with 0.075M 4°C KCl, and fixed with methanol:acetic acid (24:1 ratio) for 30 min at room temperature. Lymphocytes, in fresh fixative, were dropped into clean, iced slides, air-dried and stained with 10% Giemsa for 5–10 min at pH 7 (20).

MN count was performed on an average of 2000 binucleated lymphocytes (1000 binucleated cells per slide) with preserved cytoplasms (duplicate cultures per sample) following the criteria established by the HUMN for MN and apoptotic cells (21). The MN assay was performed blind with respect to maternal smoking status.

Testing the MN assay with BPDE

Over 4 wk, the lymphocytes from the cord blood from a further four healthy, nonsmoking mothers were also isolated as described above. These women were not included in the control nonsmoking control group and their samples were only used for the purposes of validation of the assay.

After 39 h of culture, a range of concentrations of BPDE (with THF as co-solvent) (Midwest Research Institute, Kansas City, MO) was added for a period of 5 h. Cells were then washed with PBS to remove nonincorporated BPDE, cyt-B was added, and the protocol was continued as described above.

Statistical analysis

In the design of the internal validation of the MN assay, one-way ANOVA was used to test if the mean number of MN increased with BPDE dose. The Dunnett multiple contrast test was used to evaluate differences among pairs of MN means at different BPDE doses. The nonparametric Kruskal-Wallis test was used to test whether the number of apoptotic cells varied with BPDE dose and the Mann-Whitney a posteriori test was used to evaluate differences among means of apoptotic cells at different BPDE doses.

Also, the nonparametric Kruskal-Wallis test was used to evaluate differences in the median number of MN in the smoking and nonsmoking groups. For descriptive purposes, the parametric variables are expressed as mean (SD) whereas the nonparametric are expressed as median (interquartile range).

In all cases, p values were considered significant at $p < 0.05$ and the statistical analyses were carried out with SPSS version 11.0 (SPSS, Chicago, IL).

RESULTS

The MN assay is a delicate technique that requires manipulation of cultured cells submitted to a lengthy protocol (4 d). Our approach was to set-up the standard protocol described by the HUMN project and then modify it for application to cord blood cells.

The cyt-B concentration range recommended in the literature is 3–6 μg/mL. These concentrations either yielded very few binucleated cells (3 μg/mL) or induced a cytotoxic effect on viability (6 μg/mL) so that we settled for a cyt-B concentration of 4.5 μg/mL, which was optimal for culture conditions and yielded 35–60% of binucleated cells in 500 viable cells (22) (Fig. 1).

For the final fixative stage, methanol:acetic acid in the ratio of 24:1 was used, which yielded an optimal staining of cells and nuclei while preserving nuclear and cytoplasm membrane integrity. Other ratios such as 23:2 were tried, but these resulted in paler cytoplasmic staining, which complicated MN scoring.

An internal validation of the MN assay was then designed with the intention of determining the minimal dose of BPDE that significantly increases the MN index. We initially tried BPDE in concentrations 4, 2, 1, and 0.4 μM as indicated by the available literature (23,24). These concentrations turned out to be cytotoxic for cultured lymphocytes. We then proceeded with BPDE concentrations increasing from 3.125 nM to 0.2 μM (Table 1). MN and apoptotic cells were scored at seven different BPDE concentrations (doses 1–7), without BPDE (dose 0), and with THF alone. The test was done four times over 4 consecutive weeks.

The mean number of MN increased with BPDE dose in a statistically significant manner ($p < 0.0001$, ANOVA) and the Dunnett a posteriori test showed that the minimal detectable effect was induced by dose 3, which corresponded to 12.5 nM BPDE ($p = 0.003$). No significant differences in the MN scores were detected at doses higher than 12.5 nM or between the THF control and doses from 0 to 6.25 nM BPDE (Fig. 2).

The number of apoptotic cells also statistically increased with BPDE dose ($p = 0.001$, Kruskal-Wallis test) and the Mann-Whitney a posteriori test showed that the minimal detectable effect was induced, again, by 12.5 nM BPDE ($p = 0.029$) (Fig. 2).

We detected no effect of THF on MN index or apoptotic cell score, so that the BPDE co-solvent was not per se cytotoxic for our lymphocyte cultures.

After validation, the adapted MN assay was used to determine the number of MN in cord blood samples from 102 nonsmoking mothers and 41 who smoked 10 or more cigarettes per day during pregnancy. The median (IQR) number of MN in cord blood samples from the mothers who smoked was 4 (1; 10.5), which was significantly higher than that of samples coming from nonsmoking pregnant women, 3 (0; 8) (Kruskal-Wallis, $p = 0.016$; Fig. 3).

For all cases in which mother and father gave partially contradictory answers in their questionnaires ($n = 10$), the cotinine levels in maternal urine were determined by gas chromatography–mass spectrometry, whose sensitivity is reported to be 20 ng/mL. Six out of the 10 samples tested came from nonsmoking mothers and their median cotinine concentration was 20.5 ng/mL (20; 26). The remaining four cases were classified, on the basis of their cotinine determinations, 500 ng/mL (206; 717), as belonging to the smoking group.

DISCUSSION

Tobacco consumption among women is increasing and has negative effects on fertility and gestation (3). It is estimated that between 12 and 20% of women smoke during pregnancy (4), but the exact percentage is difficult to establish because many pregnant smokers deny smoking when asked by their doctor. Indeed, one of the difficulties of this

study was to find pregnant women who were still smoking 10 or more cigarettes per day.

Although there are various studies which have found genetic instability (indicated by the presence of chromosomal aberrations, sister chromatid exchanges and/or an increased number of MN) in the peripheral blood cells of smokers (reviewed in 25,26), the effects of tobacco-specific metabolites on the fetus are poorly documented. It is known that some compounds in tobacco smoke can cross the placental barrier and the presence of tobacco-specific metabolites has been detected in the fetal fluids and the urine of fetuses and newborns (6,7). Furthermore, such compounds have been shown to induce alterations in fetal growth and development (5).

Likewise, studies that evaluate whether the frequency of MN increases in individuals who smoke are controversial, and both negative (11–13) and positive (14–16) correlations have been detected.

To our knowledge, there is only one study that specifically analyzes the frequency of MN in children (2–15 y) exposed to environmental tobacco smoke (ETS), and this study concluded that ETS-exposed children showed significantly higher MN frequencies than non-ETS-exposed children (27). In a recent meta-analysis and review of the literature, Neri and co-workers (28) reported that chromosome aberrations and MN were consistently increased in children exposed to environmental pollutants. They also conclude that in utero exposure to tobacco smoke compounds were associated with increased frequencies of DNA and Hb adducts and chromosomal aberrations. Their conclusions, as those reported herein, argue for a susceptibility of the fetus to tobacco smoke.

In a recent study, de la Chica and co-workers (8) detected a significant increase in the number of chromosomal gaps, breaks, and structural aberrations in amniocytes from women who smoked 10 or more cigarettes per day during pregnancy. The data reported here indicate increased MN in cord blood cells from such mothers ($p = 0.016$). Our results corroborate those of de la Chica and co-workers and support the hypothesis that tobacco compounds are able to induce chromosomal losses and/or breaks.

One of the main limitations in the published studies that aim to correlate the damage induced by tobacco smoke with chromatid-type chromosomal aberrations is that such aberrations are usually formed in the last round of replication before metaphase scoring and, therefore, they are formed in culture and not in the exposed individual. The MN assay detects both the chromatid and chromosome-type chromosomal aberrations and cell division is a necessary condition for MN appearance because only the acentric fragments excluded from daughter cell nuclei at telophase can produce MN (29). In some studies, the lack of correlation between results in terms of the frequencies of chromosomal aberrations and/or sister chromatid exchanges and MN (11) may be due to the fact that aberration frequencies are determined at a single point in time and are considered instantaneous mean frequencies, whereas those of MN are running means based on the cumulative number of MN derived from all divisions before the time of scoring (30).

Age is a host factor that may be involved in determining the baseline number of MN (9,10). To rule out a possible effect of age on the number of MN detected, while we

selected samples from a randomly chosen consecutive series of mothers, we excluded mothers at the age extremes, so that the smoking and nonsmoking groups did not differ in maternal age ($p = 0.547$, data not shown). In the report by de la Chica et al. (8), the mean age of the smoking group was significantly higher than that of the nonsmoking group. Nevertheless, the authors found that the increase in chromosomal aberrations was statistically significant whether maternal age was included as a covariate or not.

In our study, the overall average MN frequency was 3.5 (data not shown) which is in good agreement with previous studies that analyze the frequency of MN in healthy children (11). It is also interesting to observe that the mean MN frequency was not significantly different for male and female newborns ($p = 0.909$) and neither was it significantly different for mothers over 35 y old at time of giving birth ($p = 0.203$). In a recent review of the MN frequency in children exposed to environmental mutagens, it was concluded that the consistency of the results from the different studies argues against any clear effect of gender on MN frequency (31,32).

The MN assay is an alternative to the conventional test for chromosomal aberrations, which analyzes the alterations present in metaphase preparations. In this sense, the MN assay is useful to detect both structural (clastogenic effect) and numerical chromosomal aberrations (aneugenic effect). Other advantages of the technique are its sensitivity (given that MN are scored in cells that have completed a nuclear division), increased power of statistical testing when scoring thousands instead of hundreds of cells, and the reduced costs of the assay (33). Our results, although preliminary, support the hypothesis that smoking during pregnancy increases the genetic instability of the fetus, and that the MN assay is a valid tool to detect such an instability.

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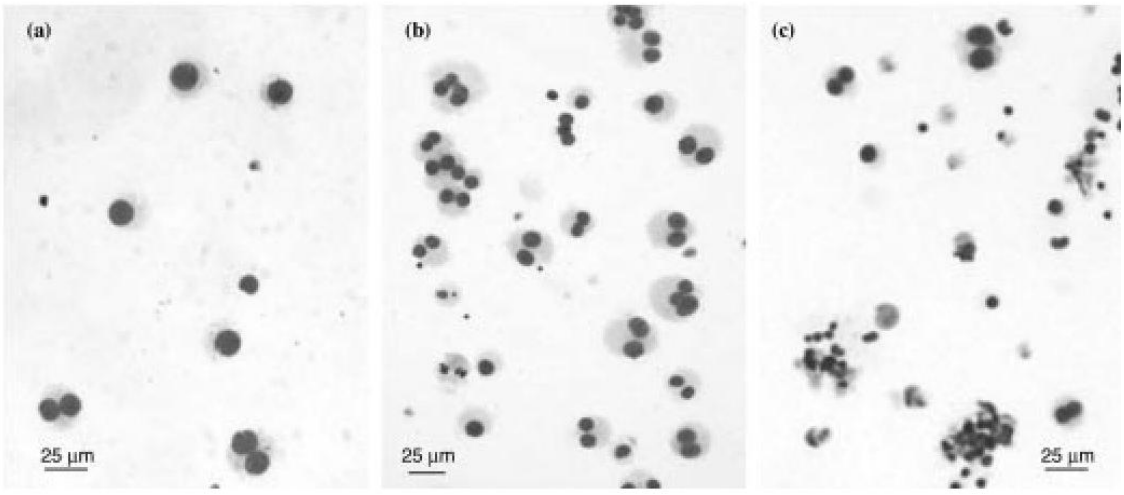

Figure 1. Cord blood lymphocyte preparations of the MN assay at 40x: (a) 3 µ/mL, (b) 4.5 µg/mL, and (c) 6 µg/mL of cytochalasin-B, respectively.

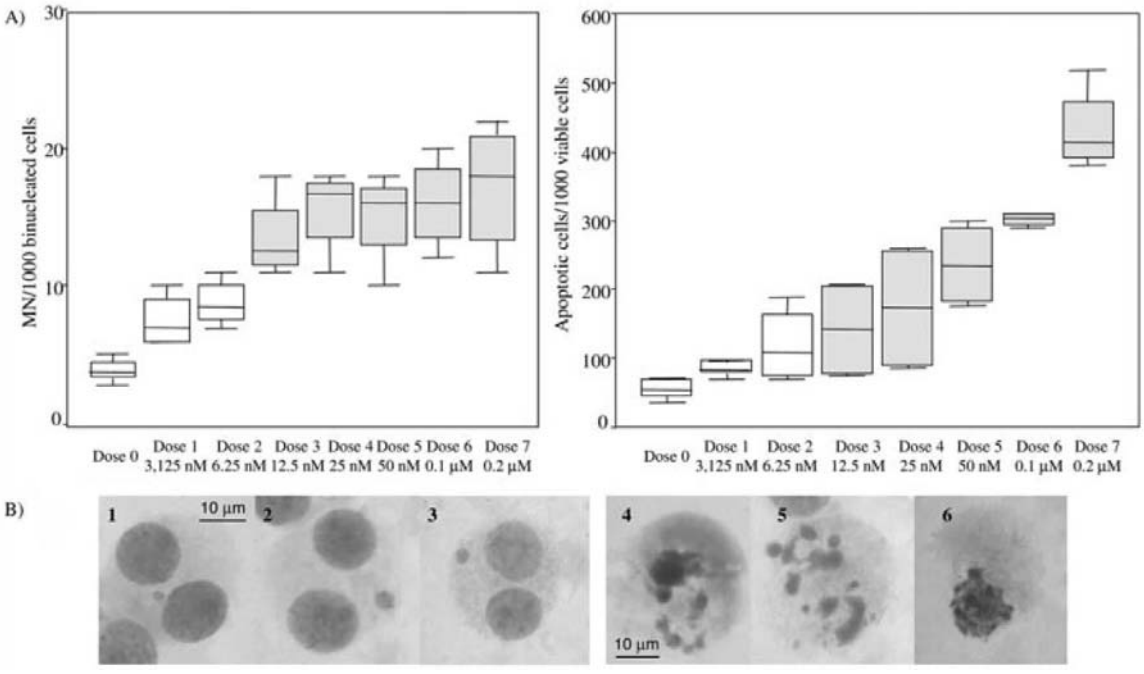

Figure 2. (*A*) Schematic representation of the MN index and number of apoptotic cells in the different BPDE doses tested. As indicated by the different fill color, the minimal detectable effect was induced by 12.5 nM BPDE, both for MN (p = 0.003) and for apoptotic cells (p = 0.029). (*B*) Microscope view of BN cells with MN (1–3) and cells in different stages of apoptosis (4–6).

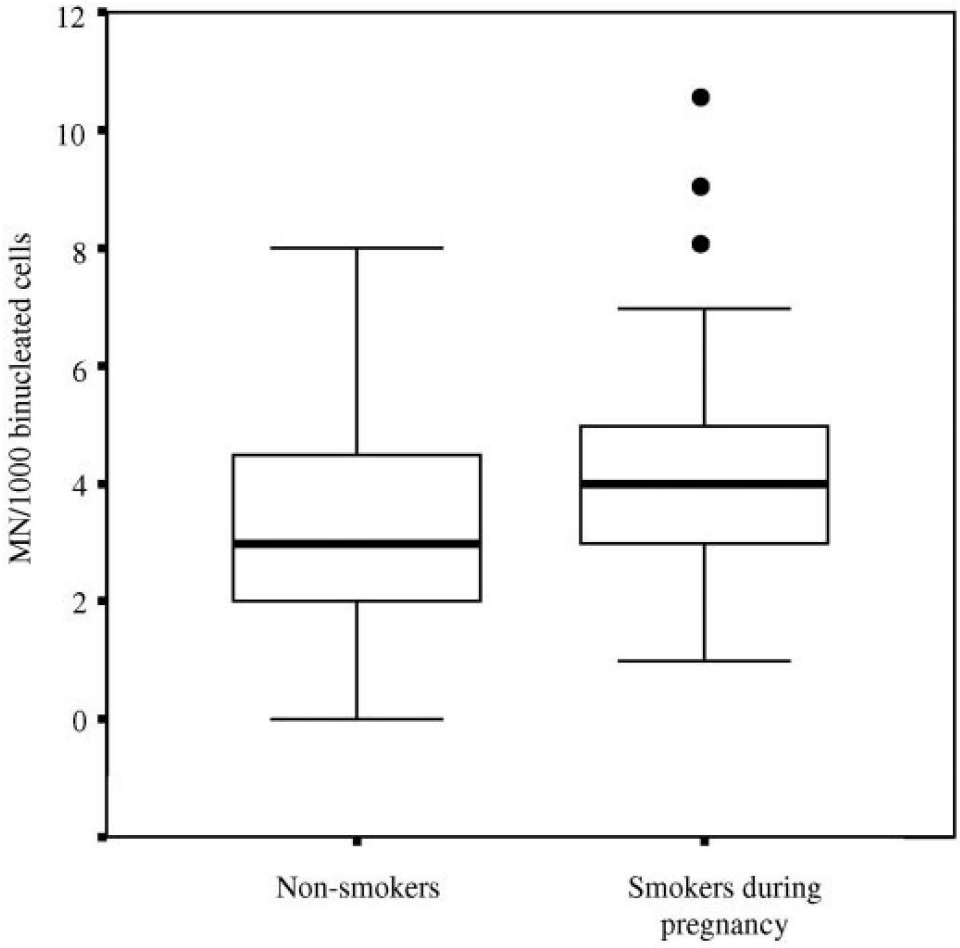

Figure 3. Comparison of the MN frequency in fetal blood samples from smoking (n = 41) and nonsmoking mothers (n = 102). The values are expressed as median and interquartile range, and outliers are marked as circles. The median number of MN in cord blood samples from the mothers who smoked was 4 (1; 10.5), which was significantly higher than that of nonsmoking pregnant women, 3 (0; 8) (Kruskal-Wallis, p = 0.016).

Table 1. Summary of MN scores and apoptotic indexes during the internal validation protocol

BPDE dose	**MN/1000 binucleated cells Mean (SD)**	**Apoptotic cells/1000 viable cells Median* (IQR)**
Negative (THF)	5 (1.22)	60.5 (51.5; 68.75)
Dose 0	4 (0.71)	61.5 (40.5; 70.5)
Dose 1 (3.125 nM)	7.5 (1.66)	89 (73.5; 95.5)
Dose 2 (6.25 nM)	8.75 (1.48)	108.5 (72.5; 175.25)
Dose 3 (12.5 nM)	13.5 (2.69)†	141.5 (76.25; 206.75)‡
Dose 4 (25 nM)	15.5 (2.69)	173 (88.25; 257)
Dose 5 (50 nM)	15 (3.00)	235 (178.75; 294.25)
Dose 6 (0.1 µM)	16 (2.92)	304 (290.5; 310)
Dose 7 (0.2 µM)	17.25 (4.21)	413 (385; 495)

The mean number of MN increased with BPDE dose and the minimal detectable effect was induced by 12.5 nM BPDE ($p < 0.0001$, ANOVA; $p = 0.003$; Dunnett *a posteriori* test). The number of apoptotic cells also statistically increased with BPDE dose, and the minimal detectable effect was induced by 12.5 nM BPDE ($p = 0.001$, Kruskal-Wallis test; ($p = 0.029$, Mann-Whitney *a posteriori* test).
* IQR, Interquartile range.