Cigarette smoke causes follicle loss in mice ovaries at concentrations representative of human exposure

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BACKGROUND: Cigarette smoke is a documented reproductive toxicant associated with infertility and ovarian failure. However, the underlying mechanism(s) regulating the toxic effects of cigarette smoke are unknown. Therefore, we tested the hypothesis that mainstream cigarette smoke and a cigarette smoke constituent, benzo[a]pyrene (BaP), induce apoptosis in ovarian follicles.

METHODS: Mice were exposed to mainstream cigarette smoke and the ovaries were analysed for follicle loss and markers of apoptosis (TUNEL, Caspase 3, Caspase 8, Bax, Bcl-2, Fas and FasL). Isolated ovaries from female pups were cultured in media containing increasing concentrations of BaP (1–10 000 ng ml−1), and markers of apoptosis were quantified.

RESULTS: Cigarette smoke exposure induced a significant reduction in the number of primordial follicles, but not growing or antral follicles compared with controls. Mainstream cigarette smoke exposure had no effect on any marker of apoptosis measured. Exposure of ovaries to BaP in vitro resulted in an increase in the pro-survival marker Bcl-2, but no change in apoptosis.

CONCLUSIONS: Our data suggest that cigarette smoke-induced follicle loss is not mediated via BaP-induced apoptosis.

Key words: benzo[a]pyrene / ovary / ovarian follicle / cigarette smoke / apoptosis

Introduction

Cigarette smoke is a documented reproductive toxicant that depletes ovarian follicle reserve and impairs uterine receptivity (Soares et al., 2007). Delayed conception (Jick and Porter, 1977; Hughes and Brennan, 1996), decreased success in assisted reproductive technologies (Klonoff-Cohen, 2005; Neal et al., 2005) and premature ovarian failure (Baird et al., 2005) have all been reported in female smokers compared with non-smokers. Although fewer Canadians are smoking today, a survey on tobacco use in Canada revealed that 14% of households reported at least one person smoked inside the home daily (Health Canada, 2006). In the Canadian Tobacco Use Monitoring Survey, 17% of female respondents report being current smokers, consuming an average of 13.8 cigarettes/day (Health Canada, 2006). What is perhaps more troubling is that young women, aged 15–19, in their reproductive prime are the fastest growing population of smokers. In southwestern Ontario alone, 36.2% of teenage girls smoke (Cohen et al., 2003) and 33% of girls are regular smokers by the age of 15, according to a British study (Augood et al., 1998). Therefore, it is imperative that we determine the mechanisms of action that explain the toxic effects of cigarette smoke on fertility.

Studies conducted in our laboratory have revealed that women exposed to cigarette smoke had greatly decreased pregnancy rates (Neal et al., 2005). We have also found that BaP is detectable in the serum and follicular fluid of women who smoke or are exposed to cigarette smoke and that treatment with BaP impairs cumulus expansion in isolated rat follicle culture experiments (Neal et al., 2007, 2008). Targeted primordial follicle destruction is considered to be the most devastating effect of cigarette smoking on reproductive function, the effects of which are not detected until years after the exposure, often after ovarian failure is well established (Cortvrindt and Smitz, 2002). Premature follicle loss has been identified as a possible causative factor for infertility. A variety of toxicants increase follicle loss and are lethal to embryos; however, the mechanism of action is unknown. Previous studies have revealed that of the more than 4000 chemicals present in cigarette smoke, levels of PAHs, especially benzo[a]pyrene (BaP), are present in levels 10-fold higher in sidestream than mainstream smoke (Lodovici et al., 2004). BaP, a member of the PAH family, a class of compounds formed by the incomplete combustion of fossil fuels and organic matter (Sagredo et al., 2006), is a ubiquitous environmental pollutant that possesses potent mutagenic properties. BaP is known to cause the formation of reproductive toxicants, among others, and is a well-known mutagen and carcinogen.
of DNA adducts and is primarily activated by P450 enzymes, most notably CYP1A1 and CYP1B1, which are regulated by the Aryl hydrocarbon receptor (AhR) pathway. Upon exposure to BaP, the AhR is bound by BaP and translocates to the nucleus, where it binds the AhR nuclear translocator and transcriptionally activates genes containing the xenobiotic response element in their promoter regions (Sagredo et al., 2006). Ovarian follicles of women exposed to cigarette smoke have detectable levels of BaP in the serum and follicular fluid (Neal et al., 2007). The follicles are also known to express the AhR (Thompson et al., 2005) and are susceptible to BaP exposure.

Numerous studies have shown that exposure to environmental toxicants (ETs) results in the destruction of the follicle population, frequently in a stage-specific manner (Devine et al., 2002, 2004; Mayer et al., 2002; Desmeules and Devine, 2006; Jurisicova et al., 2007; Neal et al., 2008). BaP has been shown to selectively target and deplete the primordial follicle pool (Mattison et al., 1980; Mattison and Nightingale, 1982). Similarly, VCD, a metabolite of 4-vinylcyclohexene and a solvent used in industry, induces apoptosis in primordial and primary follicles (Devine et al., 2002, 2004), whereas exposure to dioxin-like PCBs results in the destruction of growing follicles (Muller et al., 1978; Pocar et al., 2006), and exposure to high concentrations of non-dioxin-like PCBs, namely PCB 126 and PCB 153, results in the increased secretion of estradiol (E2) from granulosa cells and the subsequent attenuation of atretic follicle elimination (Gregoraszczuk et al., 2003). Despite the diversity of ETs shown to elicit adverse effects on ovarian function in animal models, few studies show effects in the human populations. Moreover, the decreased primary follicle numbers observed have been due to toxicological levels of these toxicants (Devine et al., 2001, 2002, 2004; Mayer et al., 2002; Takai et al., 2003; Desmeules and Devine, 2006; Jurisicova et al., 2007), high dose exposures that have debatable relevance to human exposure. Therefore, experiments were designed to determine whether cigarette smoke, at concentrations representative of human exposure, induces selective stage-dependent destruction of primordial and primary follicles in mouse ovaries.

**Materials and Methods**

**Mice for in vivo studies**

The ovarian effects of cigarette smoke exposure were studied in female C57BL/6 mice (6–8 weeks old) obtained from Charles River Laboratories (Montreal, PQ, Canada). Mice were maintained in polycarbonate cages with a 12-h light-dark cycle and unlimited access to food and tap water. All animal work described in this study was conducted using protocols approved by the McMaster University Animal Research Ethics Board and follows CCAC guidelines for the use of animals in research.

**Cigarette smoke exposure**

Mice (n = 5) were exposed to nose-only exposure whereby female mice were placed in individual exposure chambers (9 x 3 x 3 cm$^3$) and were exposed to two cigarettes daily (183 reference cigarettes; Tobacco and Health Research Institute, University of Kentucky) as described previously (Hautamaki et al., 1997). Cigarette smoke was delivered into the exposure chambers at a rate of 0.08 l min$^{-1}$. 1 puff (20 ml) per 52 s. In an initial 2-week lead-up period, mice were exposed to one cigarette in the first week and to two cigarettes in the second week. Animals were then exposed 5 days per week for a total of 8 weeks, including the 2-week lead-up period. To control for handling, groups of mice were placed in restrainers only and exposed to room air (sham exposure, n = 5). Mice were euthanized at the end of the 8-week exposure by exsanguination, and ovaries were collected and placed in Hanks’ balanced salt solution (Sigma Aldrich) prior to processing.

**Histology and immunohistochemistry**

Ovaries were fixed for standard histology, Serial sections, at 4-μm thick-ness, were stained with haematoxylin and eosin, and follicle counts were carried out as outlined in what follows. To determine the cellular localization of the active apoptotic pathways, immunohistochemical staining was performed. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) was conducted on serial sections to determine whether increased apoptosis occurred in treated versus control ovaries. Immunohistochemical staining for Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bcl-2 (Santa Cruz), Caspase 3 (Santa Cruz), Caspase 8 (AbCam, Cambridge, MA, USA), Fas (Santa Cruz) and FasL (Santa Cruz) was carried out. Briefly, following rehydration, endogenous peroxi-dase activity was quenched and antigen retrieval was carried out using citrate buffer (pH 3.0) at 37°C for 30 min. Sections were blocked with goat serum (for rabbit primary antibodies) or horse serum (for mouse primary antibodies). Avidin/biotin blocking was carried out prior to incubation with primary antibody (1:100) for 24 h at 4°C. Immunohistochemical targets were localized using diaminobenzidine (DAB; 0.25 mg ml$^{-1}$ w/v; Sigma Aldrich) in phosphate-buffered saline (PBS) and counterstained using Harris haematoxylin (Sigma Aldrich). Sections were dehydrated and cover slips were mounted using Permount. Slides were examined using an Olympus IX81 microscope at ×20 and ×40 magnification and images were captured using Image Pro AMS (Media Cybernetics, Silver Spring, MD, USA).

**Ovarian volume measurements**

Ovaries were sectioned and the number of sections recorded. This number was multiplied by the micron thickness of the sections to determine the length of each ovary. Serial sections were then measured at two radius points (width and length) and the mean of each was deter-mined. The mean radii were then used in calculating the volume of the ovary along with half the length using the formula for the volume of an ellipse: $V = \pi \times r_1 \times r_2 \times h$.

**Follicle counts**

Primordial, transitional, growing (primary and secondary) and antral follicles were identified under light microscopy using a modification of Pedersen and Peters’ classification system (Pedersen and Peters, 1968).

Briefly, primordial follicles were defined as having a single squamous cell layer surrounding the oocyte; transitional follicles as having on single cell layer of granulosa cells surrounding the oocyte whereby half the cells were squamous and half were cuboidal; primary follicles contained a single layer of cuboidal granulosa cells surrounding the oocyte; secondary follicles were any oocyte surrounded by two or more complete layers of granulosa cells and having an antrum; only follicles with a visible nucleus were counted. Every tenth section was counted from serially sectioned ovaries.

**TUNEL staining**

Serial sections were deparaffinized in xylene and rehydrated in graded ethanol solutions followed by immersion in PBS. An ApopTag® Plus
Peroxidase In Situ Cell Death Detection Kit (Chemicon International, Temecula, CA, USA) was used. Briefly, samples were treated with proteinase K and 3% H$_2$O$_2$ and labelled with digoxigenin in a humidified chamber for 30 min at room temperature. Samples were then incubated with POD-horseradish peroxidase, stained with DAB and counterstained with methyl green (0.5% w/v). Ovarian sections were examined using light microscopy as described earlier.

**DNA 1.37v software; all proteins were quantified relative to the**

**Western blotting**

Protein expression was measured in whole ovarian homogenates of either BaP-exposed ovaries or smoke-exposed and control animals. Protein was extracted from the whole ovaries using RIPA lysis buffer (2 mM v/v EDTA, 1% v/v Triton X-100, 0.1% w/v SDS, 150 mM NaCl, 0.5% w/v sodium deoxycholate), with phenylmethanesulphonyl fluoride (1 mM; Sigma Aldrich) and Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval, PQ, Canada). DNA was extracted from whole ovaries using the QIAamp DNA Mini Kit (Qiagen Sciences, MA, USA). DNA was electrophoresed on a 2% w/v agarose gel in 1 x TAE buffer at 60 V for 1 h. Gels were examined using the Epi Chem II Darkroom (UVP Bioimaging Systems, Upland, CA, USA).

**Statistical analysis**

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL, USA). Results are expressed as mean ± SEM. Data were checked for normality and equal variance and treatment effects were tested using t-test. A $P < 0.05$ was considered significant.

**Results**

**General health of animals exposed to cigarette smoke**

Animals were assessed at necropsy for changes in general health. Treatment had no effect on the general health of the mice, as shown by the absence of signs of lacrimation, porphyria or changes in body weight.

**Cotinine levels in cigarette smoke-exposed mice**

Mice exposed to cigarette smoke for 4 days had serum cotinine levels that were 220-fold higher compared with controls (mean control 0.5 ± 0.1, mean smoke-exposed group 118.9 ± 15.4). Mice normally have a 4–5-day estrus cycle, therefore an 8-week exposure would be the equivalent of approximately 11–14 cycles, the human equivalent of 1 year of uninterrupted menstrual cycles; in addition, the proposed dose is representative of a pack-a-day habit in humans, as determined previously by serum cotinine levels measured in mice exposed to this regimen.

**Effects of cigarette smoke exposure on ovarian volume**

At necropsy, gross inspection of ovaries revealed a difference in the size of cigarette smoke-exposed ovaries compared with those from age-matched sham controls (Fig. 1a). The ovarian volume of exposed mice was 20% smaller compared with sham-exposed mice, although the difference was not statistically significant ($P = 0.094$) (Fig. 1b).

**Mice for in vitro studies**

To evaluate the effect of BaP on the mechanisms underlying ovarian follicle loss, cultures of 4-day-old ovaries were employed. Briefly, male and female C57BL/6 mice (6 weeks old) were used to generate a breeding colony. A male was placed in the cage with two females for breeding. Vaginal smears were examined daily for the presence of semen, and post-coital Day 1 was assigned to the day a sperm plug was detected. Pups at 4 days postpartum were used for the in vitro ovarian organ culture studies described in what follows.

**Ovarian organ cultures**

Newborn mice were collected on post-natal Day 4. Mice were euthanized by cervical dislocation and ovaries were excised using a dissecting scope to ensure all ovarian tissue was collected. Ovaries were cultured in 2 ml of Waymouth medium 752/1 supplemented with 0.23 mM pyruvic acid, 50 mg l$^{-1}$ streptomycin sulphate, 75 mg l$^{-1}$ penicillin G, 3 mg ml$^{-1}$ BSA and 10% fetal bovine serum. Treatments with BaP (1–10 000 ng ml$^{-1}$ w/v; Sigma Aldrich, Oakville, ON, Canada; $n = 5$), vehicle control ($n = 5$) or serum-free media ($n = 5$) were carried out on Day 1 of culture for 6, 12 or 24 h. The ovaries were incubated at 37°C and infused with a 5% CO$_2$:95% air gas mixture. The media were replaced every 2 days. Ovaries were collected at the end of Day 15 and fixed for IHC, TUNEL or frozen for protein extraction.
Effects of cigarette smoke exposure on ovarian follicle numbers

Microscopic evaluation of ovarian sections revealed significant reductions in the number of follicles in different stages of development in ovaries of mice exposed to cigarette smoke for 8 weeks compared with the sham-exposed mice. Specifically, smoke-exposed ovaries had significantly fewer follicles than sham-exposed ovaries ($P = 0.01$; Fig. 2a). When the follicle numbers were further separated into follicle stage, it was evident that the primordial pool of follicles was being selectively targeted for depletion ($P = 0.04$; Fig. 2b). There were also significantly ($P = 0.04$) fewer follicles in the transitional stage (the stage between the resting primordial pool of follicles and the primary follicles in the growing pool) compared with sham-exposed mice (Fig. 2b). However, when normalized to the total number of follicles, there was a significantly ($P = 0.03$) greater percentage (7.8%) of follicles in the transitional pool of follicles of cigarette smoke-exposed mice relative to controls (Fig. 2c). Taken together, contrary to the larger proportion of follicles in the transitional pool of smoke-exposed mice compared with controls, there were no significant differences in the number of primary, secondary or antral follicles.

Cell death markers in response to cigarette smoke exposure

Treatment with 8-week cigarette smoke exposure did not result in an increase in apoptosis, as determined by TUNEL staining. There was no difference in the number of positively staining follicles between groups (Fig. 3). Positively stained follicles were defined as those with 10% or greater apoptotic granulosa cells. There were also no statistically significant differences in the apoptosis rates for each follicle type studied (data not shown). To further substantiate this finding, DNA gel electrophoresis was conducted to determine whether DNA fragmentation could be detected (Fig. 4). There was no difference in DNA fragmentation between ovaries of the cigarette smoke- and sham-exposed mice.
Protein expression was also unchanged for pro-apoptotic markers, Bax and Active Caspase 3 (Fig. 5a and b). Additional markers included Fas, FasL, Caspase 3 and Caspase 8; none showed a change in expression between treated and untreated groups (data not shown). However, the pro-survival factor, Bcl-2, was significantly ($P = 0.04$) decreased in smoke-exposed ovaries compared with sham controls (Fig. 5c).

**In vitro exposure to BaP results in decreased BcL-2 expression but not an increase in apoptosis**

*In vitro* treatment of ovaries from 4-day-old pups with BaP did not result in an increase in the expression of any of the pro-apoptotic markers tested above by western blot analysis (data not shown). Additionally, DNA fragmentation was not evident in the whole homogenates from ovaries treated with 1000 ng ml$^{-1}$ BaP for 24 h (Fig. 6). However, treatment of ovaries with 100 ng ml$^{-1}$ BaP in *in vitro* for 6 h resulted in an increase in Bcl-2 expression (Fig. 7a) and no overall change in Bax levels (Fig. 7b). Extending the treatment period to 24 h of culture with increasing concentrations of BaP produced BcL-2 and Bax protein level results consistent with the 6 h cultures (data not shown).

**Discussion**

The present study was designed to test the hypothesis that cigarette smoke exposure decreases the resting pool of follicles via increased apoptosis involving the Bax/Caspase 3 pathway. Our study demonstrates that despite significant loss of primordial follicles following exposure to cigarette smoke for 8 weeks, this loss is not attributable to apoptosis. The results of the current study contradict previous studies that have shown that exposure to toxicological levels of PAHs, chemicals present in cigarette smoke, results in follicle loss by apoptosis (Borman et al., 2000; Matikainen et al., 2001a, b, 2002). Our findings expand the literature by showing that physiologically relevant exposure to cigarette smoke does not increase the rates of apoptosis in the ovary, and by suggesting that there is an increased rate of follicle recruitment.

Serum cotinine, a metabolic breakdown product of nicotine, was significantly higher in mice exposed to cigarette smoke for 4 days, indicating that the treatment was effective in delivering CS into the systems of our test animals. Similarly, ovarian volume was visibly decreased in exposed mice and upon measurement was found to be 20% smaller than sham-exposed mice. Although this measurement was not statistically significant, it is similar to the effects of indol-3-carbinol and tamoxifen seen by Gao et al. (2002), both of which caused a decrease in ovarian weight gain. Conversely, neither VCD (Flaws et al., 1994) nor TCDD (Shirota et al., 2007) treatment results in notable changes in ovarian weight of adult ovaries. This difference could be due to the different test chemicals, doses or exposure times employed.

Despite the decrease in ovarian size, both groups exhibited follicles at all stages of development and had visible degenerating corpora lutea, as seen by microscopic inspection, indicating that ovulation was taking place. However, when individual follicles were counted, there was a significant decrease in the total number of follicles present in ovaries exposed to cigarette smoke. Previous work has attributed toxicant-induced follicle loss to apoptosis (Robles et al., 2000; Matikainen et al., 2001a, 2002; Jurisicova et al., 2007; Kim

![Graph A](image1.png)

**Figure 2** The number of follicles was determined in serial sections of ovaries from sham ($n = 5$) and cigarette smoke-exposed ($n = 5$) mice. (A) The total follicle number in ovaries from smoke-exposed mice was significantly ($P < 0.016$) lower than in sham-exposed mice. (B) Ovaries from smoke-exposed mice had significantly fewer primordial ($P = 0.01$) and transitional ($P = 0.04$) follicles than the sham exposure group. (C) Ovaries from smoke-exposed mice had a significantly ($P = 0.03$) higher percentage of follicles in transition between the resting and growing pools than ovaries from the sham exposure group. Overall treatment effects were determined by t-test.
et al., 2008). In the study conditions explored here, however, no apparent increase in apoptosis was detected. TUNEL assays revealed that there was an equivalent amount of apoptosis occurring in smoke-exposed ovaries as was taking place in sham ovaries. In addition, electrophoresis gels run to examine the extent of DNA laddering failed to show an increase in apoptosis in any of the treatments administered, in vivo or in vitro. Finally, western blot analysis of the expression of proteins previously shown to be up-regulated in ovaries treated with toxicants active Caspase3 (Devine et al., 2002; Desmeules and Devine, 2006), Bax, Caspase 2 and Caspase 3 (Takai et al., 2003) was unaffected by treatments in the current study. Our findings suggest that a decreased growth support leading to an abbreviated estrous cycle and thus an enhanced rate of follicle recruitment may be the underlying cause of follicle demise rather than apoptosis. In previous experiments carried out in our laboratory, inclusion of BaP in the culture medium of in vitro-isolated rat follicle culture resulted in the inhibition of follicle growth. BaP at 1.5 ng ml$^{-1}$, a concentration representative of levels measured in human ovarian follicular fluid, resulted in the failure of cumulus cells to expand compared with controls (Neal et al., 2007). Similarly, treatment of isolated follicles in vitro with BaP resulted in a concentration-dependent decrease in E$_2$ concentrations in spent media. Disruption of E$_2$ production may be responsible for the lack of follicle growth contributing to a shorter estrous cycle, allowing more cycles/year thereby ageing these ovaries faster than sham ovaries. This hypothesis is supported by the lack of an increase in the number of growing follicles.

Further support for this hypothesis is derived from epidemiological studies in women who smoke whose menstrual cycles are also shortened (Windham et al., 1999).

Contrary to the in vivo studies, in vitro dosing of 4-day-old ovaries resulted in a change in Bcl-2 expression. When ovaries were incubated for 6–24 h in media containing 100 ng ml$^{-1}$ of BaP, the expression of Bcl-2 was increased, although Bax expression was unchanged overall. Thus a shift in the Bax:bcl-2 ratio was seen, resulting in an environment that favours survival. This change therefore cannot account for the loss of follicles in vitro. It is also important to note that 100 ng ml$^{-1}$ BaP is a concentration that we believe to be much greater than those achieved in vivo. The concentration of BaP in the serum and follicular fluid of women who smoke (1.32 $\pm$ 0.68 ng ml$^{-1}$) or are exposed to secondhand smoke (0.05 $\pm$ 0.01 ng ml$^{-1}$) is significantly lower than the concentrations used (100 ng ml$^{-1}$) in this experiment. Furthermore, we have shown that concentrations equivalent to the levels measured in human serum and follicular fluid are sufficient to impair follicle expansion and survival in individual follicle culture experiments (Neal et al., 2007, 2008). However, it is likely that the difference in dose required to detect a response is related to a number of factors. First, individual follicles in culture are surrounded by a thecal cell layer only, whereas follicles in intact ovaries used in our experiments here are surrounded by stroma and other follicles. Additionally, the intact ovaries have a tunica surrounding the ovary, a tough membrane that likely obstructs BaP in the media from reaching the follicles within the ovary. Hence, the actual concentration of BaP capable of exerting an effect on the follicles within the intact ovary may be much lower than 100 ng ml$^{-1}$. Follicular fluid measurements of BaP were not conducted in this study, and as such, this hypothesis cannot be tested at present. Future studies will include measurement of BaP in the follicular fluid of intact ovaries cultured in BaP-containing media. To determine whether BaP is reaching the ovary, future work will include serum measures of BaP and testing for the formation of DNA adducts in the ovaries of exposed mice.

**Figure 3** Representative photomicrographs of TUNEL-stained ovarian sections for apoptosis. There was no difference in the number of apoptotic follicles in ovaries from the (A) sham (n = 5) compared with (B) smoke-exposed (n = 5) mice.

**Figure 4** There was no detectable increase in apoptosis as determined by DNA laddering in ovaries from smoke-exposed (n = 5) compared with ovaries from sham-exposed mice (n = 5). Marker sizes are depicted in base pairs.
The findings of our study show that exposure to cigarette smoke, at exposure concentrations representative of human exposure, results in a significant primordial follicle loss. This loss, however, does not appear to be due to apoptosis, as has been shown to be the case when toxicological levels were employed. Our data provide further support to a growing body of evidence that cigarette smoke is a reproductive toxicant that results in premature ovarian failure.

**Figure 5** Western blot analysis of pro-apoptotic and pro-survival proteins was performed on whole ovary homogenates from sham-(n = 5) and smoke-exposed mice (n = 5). Expression of (A) Bax and (B) active Caspase 3 was unchanged in 8-week exposed mice; however, (C) Bcl-2 levels were significantly decreased in exposed mice compared with age-matched controls (P = 0.04).

**Figure 6** Representative gel of DNA laddering for apoptosis in in vitro studies. There was no change in apoptosis in BaP-treated ovaries (1000 ng ml⁻¹ for 24 h; n = 5) compared with control ovaries (n = 5). Marker sizes are depicted in base pairs.

**Figure 7** Representative immunoblots prepared from homogenates of 4-day-old ovaries demonstrating (i) an increase in Bcl-2 protein levels and (ii) no consistent change in Bax protein levels following treatment with increasing concentrations of BaP (lanes 1–6 are 0, 1, 10, 100, 1000 and 10 000 ng BaP/ml) for 6 h. Densitometric analysis of immunoblots was performed and Bcl-2 and Bax protein expression levels were quantified relative to the β-actin loading control with the corresponding results shown in the accompanying graphs.

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