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ORIGINAL ARTICLE *Topic*

Comparison of toxicity of smoke from traditional and harm-reduction cigarettes using mouse embryonic stem cells as a novel model for preimplantation development

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BACKGROUND: Embryonic stem cells (ESC), which originate from the inner cell mass of blastocysts, are valuable models for testing the effects of toxicants on preimplantation development. In this study, mouse ESC (mESC) were used to compare the toxicity of mainstream (MS) and sidestream (SS) cigarette smoke on cell attachment, survival and proliferation. In addition, smoke from a traditional commercial cigarette was compared with smoke from three harm-reduction brands.

METHODS: MS and SS smoke solutions were made using an analytical smoking machine and tested at three doses using D3 mESC plated on 0.2% gelatin. At 6 and 24 h, images were taken and the number of attached cells was evaluated.

RESULTS: Both MS and SS smoke from traditional and harm-reduction cigarettes inhibited cell attachment, survival and proliferation dose dependently. For all brands, SS smoke was more potent than MS smoke. However, removal of the cigarette filter increased the toxicity of MS smoke to that of SS smoke. Both MS and SS smoke from harm-reduction cigarettes were as inhibitory, or more inhibitory, than their counterparts from the traditional brand. When preimplantation mouse embryos were cultured for 1 h in MS or SS smoke solutions from a harm-reduction brand, blastomeres became apoptotic, in agreement with the data obtained using mESC.

CONCLUSIONS: mESC provide a valuable model for toxicological studies on the preimplantation stage of development and were used to show that MS and SS smoke from traditional and harm-reduction cigarettes are detrimental to embryonic cells prior to implantation.

Key words: embryonic stem cells / cigarette smoke / development / embryos / reproduction

Introduction

Embryos and fetuses are generally more sensitive to environmental toxicants than adults, and there is a recognized need for new assays to study the effects of environmental toxicants on prenatal stages of development (Grandjean *et al*., 2007). Since 'time' as much as 'dose' determines chemical susceptibility *in utero*, it is important to develop assays that can monitor the effects of environmental

chemicals at different times during prenatal development, including the preimplantation stage. Because embryonic stem cells (ESC) are derived from the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981), they represent a powerful *in vitro* model for studying the earliest stages of mammalian development. In this study, we have used ESC as a toxicological model to assess the effects of cigarette smoke on preimplantation development.

Cigarette smoke was chosen as the toxicant for evaluation as it is known from numerous epidemiological studies that mainstream (MS) cigarette smoke, which is actively inhaled by smokers, can lead to a variety of adverse reproductive outcomes that include spontaneous miscarriage, placenta abruption, perinatal mortality, congenital malformations, ectopic pregnancy, length of time to conceive, and decrease in birthweight and fertility rate (Shiverick and Salafia, 1999; Andres and Day, 2000; Higgins, 2002; Berthiller and Sasco, 2005; Rogers, 2008). More recently, post-natal defects in cognition, intellectual development and behavior, as well as adverse respiratory effects, have been correlated with MS cigarette smoke exposure during pregnancy (DiFranza *et al*., 2004; Jaakkola and Gissler, 2004; Lannero *et al*., 2006; Perera *et al*., 2006). Although not as thoroughly studied as MS smoke, epidemiological evidence also indicates that sidestream (SS) smoke (the smoke that burns off the end of a cigarette) can adversely affect reproduction, e.g. by reducing birthweight (Windham *et al*., 1999; Hruba and Kachlik, 2000; Goel *et al*., 2004; Hegaard *et al*., 2006) and increasing fetal mortality and preterm delivery (Kharrazi *et al*., 2004).

A number of *in vitro* tests have been developed to study how cigarette smoke interacts with the reproductive organs and affects prenatal development (Talbot, 2008). These *in vitro* assays, which allow rapid testing of cigarette smoke and its components on the reproductive system using controlled conditions, have identified the ovaries, uterus and oviduct as targets of cigarette smoke (Shiverick and Salafia, 1999; Mlynarcikova *et al*., 2005; Talbot and Riveles, 2005; Neal *et al*., 2007; Talbot, 2008). *In vitro* culture of post-implantation embryos has been used to show that nicotine, a major component of tobacco smoke, retards growth in the brain and branchial arches of rats (Joschko *et al*., 1991) and induces apoptosis in both the brain and spinal cord of mice (Zhao and Reece, 2005). Recently, the 'embryonic stem cell test' was introduced to monitor the effects of chemicals on embryoid bodies derived from mouse ESC (mESC) (zur Nieden *et al*., 2004; Seiler *et al*., 2006). This assay, which models post-implantation development, is a good predictor of chemicals that are teratogenic. In the current study, mESC were used as a model to test the toxicity of cigarette smoke on preimplantation development.

Most prior work on cigarette smoke has been done using either research (e.g. 2R1 or 1R4F) or traditional (full-flavored) commercial (e.g. Marlboro Reds) brands of cigarettes. 2R1 (high-tar unfiltered) and 1R4F (lower tar and filtered) research cigarettes were manufactured by the University of Kentucky to provide researchers with standardized cigarettes for testing. In addition to traditional brands such as Marlboro Reds, cigarette companies now market 'harm-reduction' brands (e.g. Marlboro Lights, Advance Lights and Quest), which claim to have reduced levels of toxicants, in particular carcinogens. Harm-reduction cigarettes are manufactured by incorporating ventilation holes into filters to dilute smoke before it is inhaled (e.g. Marlboro Lights), using alternate curing processes to reduce tobacco-specific nitrosamines (e.g. Advance Lights), adding chemicals such as palladium to the tobacco leaves to reduce tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons, both carcinogens (e.g. Omni), and genetically engineering the tobacco plant to significantly reduce nicotine concentration (e.g. Quest) (http://www.apa.org/science/psa/sb-hatsukami.html). Cigarette companies have generally claimed that harm-reduction cigarettes are not as dangerous to smokers' health as traditional brands (http://www.apa.org/science/psa/sb-hatsukami.html). However, it is now known that harm is not reduced in the case of 'light' or 'low-yield' cigarettes. To obtain adequate concentrations of nicotine, smokers of 'light' cigarettes (a type of harm-reduction product) inhale larger puffs, smoke more cigarettes and smoke down close to the butt thereby making their exposure equivalent to or greater than smokers of traditional 'full-flavored' cigarettes (Warner, 2005).

Compared with traditional and research brands, there are relatively few studies on harm-reduction cigarettes and their effects on reproduction. It has been shown that MS and SS smoke, from both traditional and harm-reduction cigarette smoke, inhibit ciliary beat frequency, oocyte collection rate and smooth muscle contractions of the hamster oviduct (Riveles *et al*., 2007). These data indicate that while harm-reduction cigarettes may have reduced the levels of carcinogens, MS and SS smoke from these products still retains toxicants that are inhibitory in diverse biological assays involving the oviduct. The purpose of this study was to compare the toxicity of MS and SS smoke from traditional and harm-reduction cigarettes using ESC as a model for preimplantation embryos.

Materials and Methods

Chemicals and tissue culture supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, L-glutamine and β-mercaptoethanol were purchased from Sigma Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), 100 mM sodium pyruvate and 1 × trypsin/EDTA were purchased from Gibco (InVitrogen, Carlsbad, CA, USA). Non-essential amino acids were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Leukemia inhibitory factor (LIF) was purchased from Chemicon International (Temecula, CA, USA). Tissue culture flasks (T-25 and T-75) were from Nunc (Fisher Scientific, Tustin, CA, USA). Tissue culture plates (35 × 100 mm) were from Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Phosphate-buffered saline was made using deionized water, autoclaved, and stored at 4°C. Research cigarettes (2R1 and 1R4F) were purchased from the University of Kentucky. Commercial brand cigarettes were purchased from retail dealers, and these included Marlboro Red (filter cigarettes, tar = 15 mg, nicotine = 1.1 mg) and Marlboro Lights (filter cigarettes, tar = 10 mg, nicotine = 0.8 mg) from Philip Morris Inc. (Richmond, VA, USA); Advance Premium Lights 100s (filter cigarettes, tar = 10 mg, nicotine = 0.8 mg) from Brown and Williamson Tobacco (Louisville, KY, USA); Quest (filter cigarettes, tar = 10 mg, nicotine ≤ 0.05 mg) from Vector Tobacco Inc. (Mebane, NC, USA).

Preparation of smoke solutions

MS and SS smoke solutions were prepared using a University of Kentucky analytical smoking machine. MS smoke solutions contain tobacco toxicants inhaled by active smokers, whereas SS smoke solutions contain chemicals inhaled by passive smokers. Both MS and SS smoke solutions were made in DMEM medium, and 10 cigarettes were used to achieve the

concentration of 100 puffs of smoke dissolved in 5 ml of medium (10 puffs/cigarette). Concentrations of smoke solution were measured in puff equivalents (PE). PE is defined as the number of puffs of cigarette smoke dissolved in 1 ml of aqueous solution (1 PE=the smoke from one puff that dissolves in 1 ml of medium). Serial dilutions were performed to achieve the PEs desired. All experiments were done using 0.0PE (control), 0.01PE, 0.1PE or 1.0PE of either MS or SS smoke from research, traditional (Marlboro Red) or harm-reduction (Marlboro Lights, Advance Lights and Quest) cigarettes. In some experiments, MS smoke solutions were made after removing the filter from cigarettes.

Cell cultures

Mouse embryonic fibroblasts (mEFs) were isolated from 13.5-day-old embryos using the ATCC protocol. Fibroblast cultures were expanded on 0.2% gelatin-coated Nunc T-25 flasks (Fisher Scientific) and irradiated with cesium^{137} (8000 rads for 126.25 min) at passage 3 when cultures were 90–95% confluent. mEF culture medium was changed to regular ESC medium for at least 1 h prior to stem cell plating.

D3 mESC were purchased from ATCC (#CRL-11632, Manassas). All experiments were done with passages 9–24. D3 mESC were plated on mitotically inactivated mEFs in stem cell medium containing 81.5% DMEM, 15% FBS, 0.98% L-glutamine, 0.98% sodium pyruvate, 0.98% non-essential amino acids, 0.5% penicillin/streptomycin, 0.00065% β-mercaptoethanol and 0.00025% LIF. The medium was changed daily, and cell confluency was also examined. Cells were used for experimentation or frozen down at 70–75% confluency. Stem cell cultures were used in experiments 48–72 h after plating. All cultures were maintained in a 37°C, 5% CO_2 incubator.

Animals

National Institutes of Health (NIH) Swiss white mice, purchased from Harlan (San Diego, CA, USA), were housed in the University of California, Riverside vivarium. The mice were on a 14-h light and 10-h dark cycle, and they were fed Purina rodent chow (Ralston-Purina, St Louis, MO, USA). Animal protocols were approved by the Campus Committee on Animal Care.

Collection of preimplantation embryos

For collection of preimplantation embryos, NIH Swiss white mice were superovulated and mated. To induce ovulation induction, mice were injected i.p. with 10 IU of pregnant mare's serum gonadotrophin at 1430 h followed by 10 IU of HCG after 46 h. The female mice were then placed in cages containing two male mice. Preimplantation embryos were collected 3 days after mating by flushing the oviducts with mESC medium.

mESC attachment, survival and proliferation assay

To examine the effects of cigarette smoke on mESC attachment, survival and proliferation, experiments were done over 24 h. mESCs on mEF feeder layers were detached using 0.05% trypsin. In each experiment, mESCs were separated from fibroblasts by plating on 0.2% gelatin-coated 60 mm dishes. After 25 min, the supernatant containing mainly mESC was collected, and the procedure was repeated again. After mESC isolation, the number of cells needed for each sample was determined using a hemacytometer. Tissue culture dishes (35 mm) were coated with gelatin, and mESCs were plated at 100 000 cells per dish in medium containing varying doses of smoke solutions. Images were taken at 0, 6 and 24 h, and the number of attached cells was determined at 6 and 24 h.

Effect of smoke solutions on pre-attached mESC

To determine the effect of smoke solutions on pre-attached mESC, cells were plated on gelatin-coated dishes (100 000 cells per dish) for 6 h to allow maximum attachment. Cells were then treated with Advance MS or SS smoke (0.0PE, 0.01PE, 0.1PE or 1.0PE) for 24 h, at which time the number of attached cells was counted.

Pretreatment of mESCs with cigarette smoke

mESCs, separated from the fibroblasts, were incubated in 0.0PE or 1.0PE of Advance MS or SS smoke solution for 1 h in low-attachment dishes (Corning Inc., Corning, NY, USA) at a density of 100 000 cells per plate. After treatment, stem cells were collected, centrifuged, resuspended in fresh medium without smoke solution, and re-plated on 0.2% gelatin-coated 35 mm dishes in medium that did not contain smoke solutions. Images and the number of attached cells were recorded at 6 and 24 h.

Apoptosis detection assay

To determine if cigarette smoke induced apoptosis in mESC and preimplantation embryos, FLICA Caspase Detection Kits (Immunochemistry Technologies, LLC, Bloomington, MN, USA) were used to stain mESC or preimplantation embryos for activated caspases. mESC were treated with MS or SS cigarette smoke for 6 h, then incubated in the polycaspase-FLICA^{TM} for 30 min to detect polycaspase activity. Preimplantation embryos were incubated in MS or SS smoke solution for 1 h, then treated with SR-DEVD-FMK Caspase 3&7 FLICA^{TM} reagent for 30 min. After staining with FLICA reagents, cells or embryos were washed three times for 20 min using the washing buffer provided with the kit, then samples were mounted on glass slides and viewed using a Nikon fluorescent microscope.

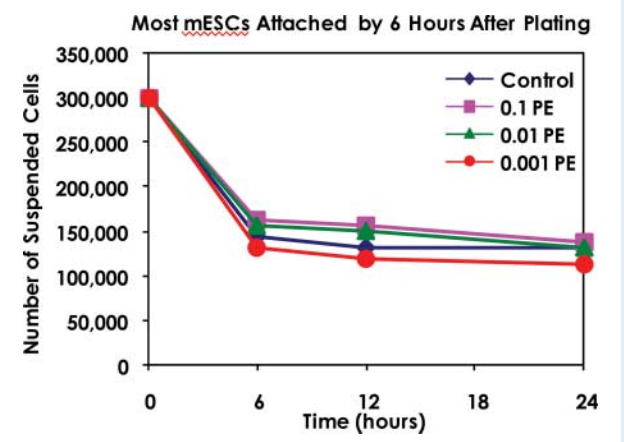

Figure 1 Representative experiment showing the number of suspended mouse embryonic stem cells (mESC) at various times of incubation in control medium or various concentrations of mainstream (MS) smoke from 2R1 research cigarettes.
Since the number of suspended cells remained unchanged in all groups after 6 h, cell attachment was interpreted to be complete by this time.

Statistical analysis

Statistical significance was evaluated using a one sample *t*-test to compare the control group (untreated), which was set to 100%, with the mean percentage of each treatment group for each endpoint assay. GraphPad (GraphPad, San Diego, CA, USA) was used for all statistical analyses. Means were considered to be significantly different for $P < 0.05$.

Results

Basis for mESC attachment assay

The purpose of the first experiment was to determine the optimal time for quantifying mESC attachment to 0.2% gelatin. Preliminary trials suggested that mESC attached during the first 6 h of incubation (not shown). To test this more rigorously, cells in control medium or in various doses of MS or SS smoke solution were plated, and the number of suspended cells was counted at 6, 12 and 24 h. The number of suspended cells in both MS (Fig. 1) and SS (not shown) smoke solution leveled off after 6 h in all groups indicating that attachment was complete by this time. In subsequent experiments, attachment was compared at 6 h, the time of maximum attachment.

MS and SS smoke solutions from traditional and harm-reduction cigarettes inhibited mESC attachment dose dependently

Traditional (Marlboro Red) and harm-reduction (Marlboro Lights, Advance and Quest) MS and SS cigarette smoke solutions were tested for their effects on mESC attachment at 6 h, using three doses (0.1, 0.01 and 1.0PE). For each cigarette brand, the number of attached cells was plotted relative to the control, which did not contain smoke solution (Fig. 2 A–D). MS (filtered and non-filtered) and SS smoke solutions from traditional and harm-reduction cigarettes significantly inhibited mESC attachment dose dependently. In all four brands of cigarettes, non-filtered MS smoke was more inhibitory than filtered MS smoke, showing that the filter removed toxicants. In general, SS smoke and non-filtered MS smoke had similar inhibitory

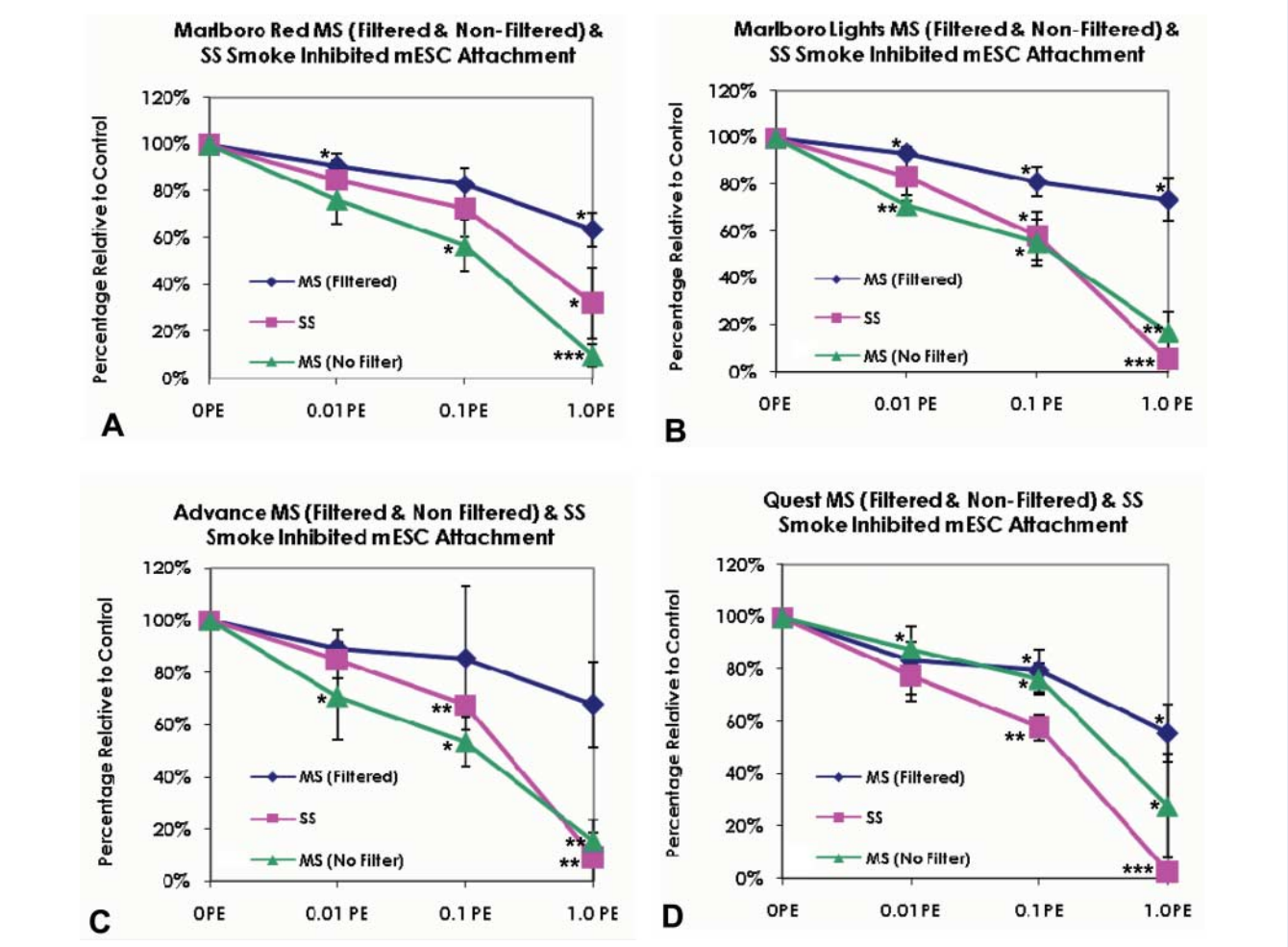

Figure 2 Traditional and harm-reduction MS and sidestream (SS) smoke inhibited mESC attachment dose dependently.
Data are shown for four groups of cigarettes: (**A**) Marlboro Red (traditional), (**B**) Marlboro Lights (harm reduction), (**C**) Advance (harm reduction) and (**D**) Quest (harm reduction, nicotine-free). Each point is the mean ± SD of three experiments. Statistical significance was determined using a one-sample *t*-test in which smoke treated groups were compared with a hypothetical mean of 100% (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

activity. Attachment was also inhibited dose dependently by MS and SS smoke from 2R1 and 1R4F research brand cigarettes (not shown).

Basis for evaluating death, survival or proliferation of smoke-treated mESC

To determine if attached mESC die, survive or proliferate in the presence of smoke solutions, the number of attached cells was counted at 24 h and compared with values at 6 h. Three possible outcomes were observed as shown in Fig. 3. Relative to the number of attached cells at 6 h, cell number at 24 h either increased (proliferation occurred), stayed the same (cells survived) or decreased (cells that had been attached died).

MS and SS smoke inhibited mESC proliferation and survival dose dependently

Filtered MS smoke solutions from the four brands of cigarettes were tested for their effects on mESC proliferation (Fig. 4A–D). Filtered MS smoke from all brands of harm-reduction cigarettes significantly inhibited proliferation dose dependently. In contrast, filtered MS smoke from Marlboro Red traditional cigarettes did not have a significant effect at any dose. At 1.0PE, the highest dose tested, MS smoke from the harm-reduction brands either caused cell death (Marlboro Lights) and or enabled survival without significant proliferation (Advance and Quest). MS smoke from 2R1 and 1R4F research cigarettes produced results similar to Quest cigarettes, but were both somewhat more potent at the 0.1PE dose (not shown).

When similar experiments were done using cigarettes from which the filter had been removed, non-filtered MS smoke from all four brands had similar effects on mESC survival and proliferation. The highest dose (1.0PE) of smoke from each brand killed all cells (Fig. 5A–D). At 0.1PE, cells were able to survive, but did not proliferate significantly. All non-filtered MS smoke solutions (Fig. 5) were more potent than filtered MS smoke (Fig. 4).

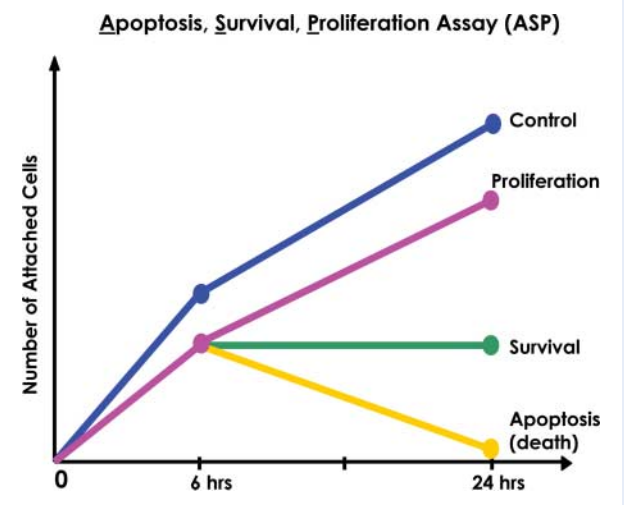

Figure 3 Basis for evaluating death, survival or proliferation of smoke-treated mESC.
Schematic diagram demonstrates the three possible outcomes at 24 h when compared to the 6 h sample: (1) increase in cell number (proliferation), (2) no change in cell number (survival, but not proliferation) and (3) decrease in cell number (death).

SS smoke solutions from all four cigarette brands showed detrimental effects on mESC survival and proliferation (Fig. 6A–D). At 1.0PE, cells did not survive in any treatment group. At 0.1 PE, cells died in SS smoke from Marlboro Light cigarettes, survived in smoke from Quest and proliferated at a slower rate than the control in Marlboro Red and Advance SS smoke. In general, SS smoke and non-filtered MS smoke were similar in their effects on mESC survival and proliferation, while filtered MS smoke was the least potent of the three types of smoke tested. SS smoke from 2R1 and 1R4F research cigarettes produced results similar to Marlboro Lights (not shown).

To demonstrate that the higher doses of smoke induced apoptosis in mESC, cells were labeled with an inhibitor that fluoresces red when bound to activated caspases (polycaspase-FLICA). After 6 h of incubation, 93% of the attached cells treated with 1.0 PE of SS smoke solution from 2R1 cigarettes were labeled with activated polycaspase inhibitor, while only 1% of the cells were apoptotic in the untreated control (not shown).

Smoke treatment caused loss of pre-attached cells

In all the previous experiments, mESC were plated in the presence of smoke solutions. The purpose of this experiment was to determine if cells that had attached prior to MS or SS smoke treatment would be protected from the effects of smoke solutions. mESC were plated on gelatin-coated dishes in standard mESC medium, and attachment was allowed to occur. At 6 h, stem cells were treated with different doses of Advance MS (filtered) or SS smoke, and the number of attached cells in each group was counted 24 h after treatment. In both MS and SS groups, the number of pre-attached cells decreased significantly with increasing smoke concentrations (Fig. 7A and B). Moreover, all attached cells in 1.0PE of SS smoke were detached from the plates after 24 h of treatment. In agreement with previous experiments, SS smoke was more potent than MS smoke for all doses.

Smoke pretreatment of mESC inhibited attachment and proliferation

To determine if mESC can be rescued from smoke solution treatment, cells were pre-treated with Advance MS (filtered) or SS smoke for 1 h in low-attachment dishes then re-plated onto gelatin-coated dishes with fresh medium that did not contain smoke solution (Fig. 8A and B). At 6 and 24 h, the number of attached cells was determined. Pretreatment of mESC with Advance MS or SS smoke solutions significantly inhibited attachment at 6 h. At 24 h, attached cells in MS smoke solutions were able to proliferate, but at a slower rate than in the control group. In SS smoke solution, the number of attached cells at 24 h decreased slightly when compared with the 6 h group.

Smoke treatment induced apoptosis in preimplantation mouse embryos

Since mESC represent a proxy for the inner cell mass of preimplantation embryos, the effect of smoke solutions on actual mouse embryos was tested. Preimplantation embryos were treated with 0.1PE of Advance MS or SS smoke for 1 h then stained with a FLICA reagent that detects activation of caspases 3 and 7. In smoke-treated

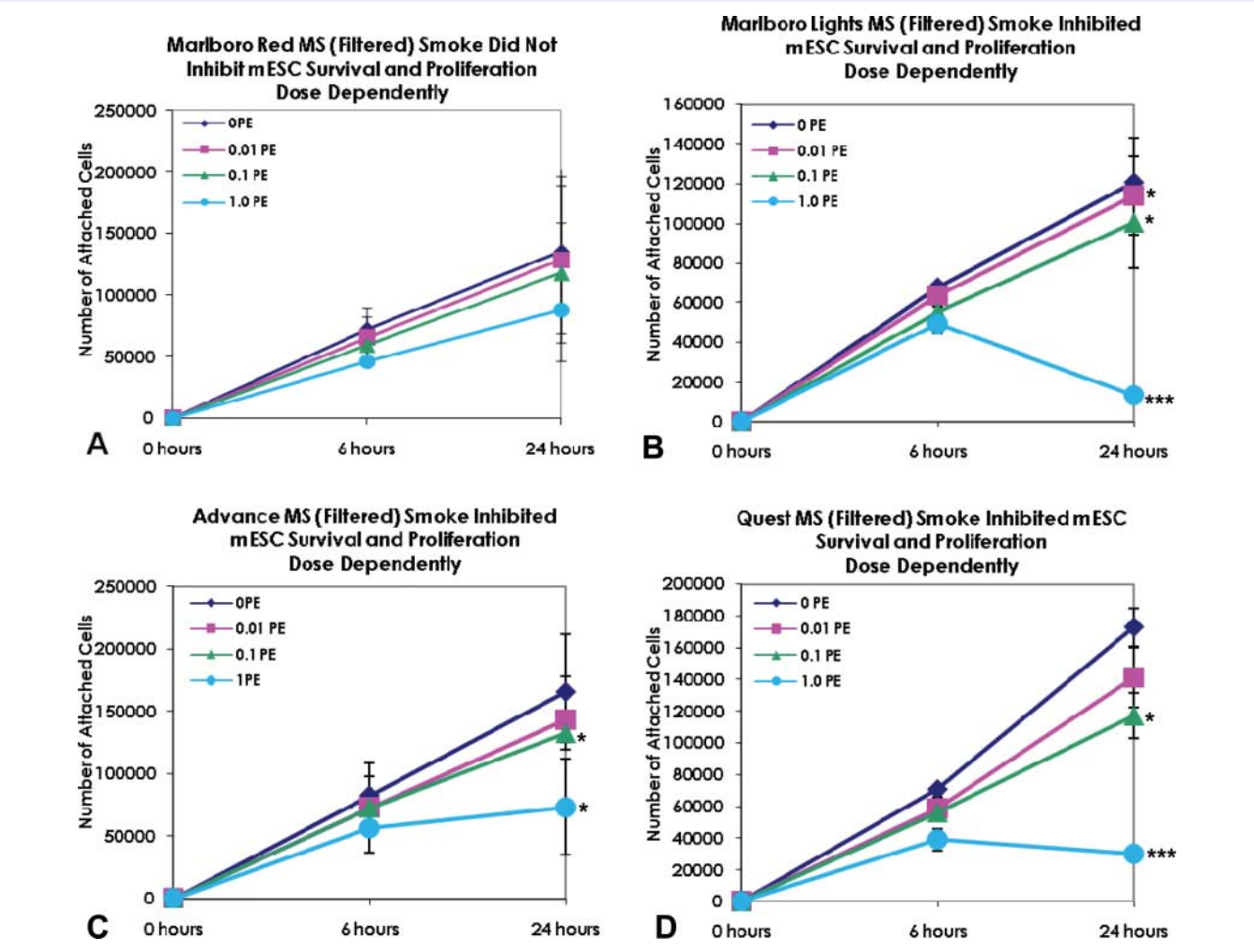

Figure 4 Filtered MS smoke inhibited survival and proliferation of mESC dose dependently.
(**A**) Marlboro Red filtered MS smoke, (**B**) Marlboro Lights filtered MS smoke, (**C**) Advance Lights filtered MS smoke and (**D**) Quest filtered MS smoke. Each point is the mean ± SD of three experiments. Statistical significance was determined using a one-sample *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

embryos, some blastomeres were morphologically abnormal and were degenerating compared with the control (Fig. 9A–C). In addition, blastomeres in smoke-treated embryos were positive for activated caspases 3 and 7, while control blastomeres were negative (Fig. 9D–F).

Discussion

We have developed and used a straightforward rapid *in vitro* assay based on attachment, proliferation, survival and death of mESC to compare the toxicity of traditional and harm-reduction tobacco smoke. Because mESC originate from the inner cell mass of blastocysts, this assay models the effects of smoke on preimplantation development. For all endpoints (cell attachment to a gelatin, survival, proliferation and death), harm-reduction cigarette smoke (Marlboro Lights, Advance Lights and Quest) was as potent as, or more potent than, traditional cigarette smoke (Marlboro Red). In addition, our data showed that for all brands tested, SS smoke solutions were considerably more potent than filtered MS smoke solutions. However, removal of the filter increased the toxicity of MS smoke to the levels observed for SS smoke. Pre-attachment of cells to gelatin did not protect them from the effects of MS or SS smoke solutions. Moreover, pretreatment of cells with smoke solution for only 1 h before plating was sufficient to reduce attachment, proliferation and survival when cells were subsequently plated in medium that did not contain smoke solution, indicating that relatively short exposures to smoke are sufficient to induce harm in mESC and that harm was not readily reversible. Mouse preimplantation embryos behaved similarly to mESC when exposed to smoke solutions (they showed degenerating blastomeres), supporting the idea that mESC are a valid model for preimplantation embryos. These data are consistent with the idea that cigarette smoke is toxic to preimplantation embryos and can retard growth or kill embryonic cells at this stage of development.

Attachment of cells to each other and to extracellular matrices is necessary for normal embryonic development. When mESC were plated in the presence of smoke solutions, attachment to gelatin was inhibited by both MS and SS smoke from each brand of cigarette tested. Pre-attachment of cells to gelatin did not protect them from

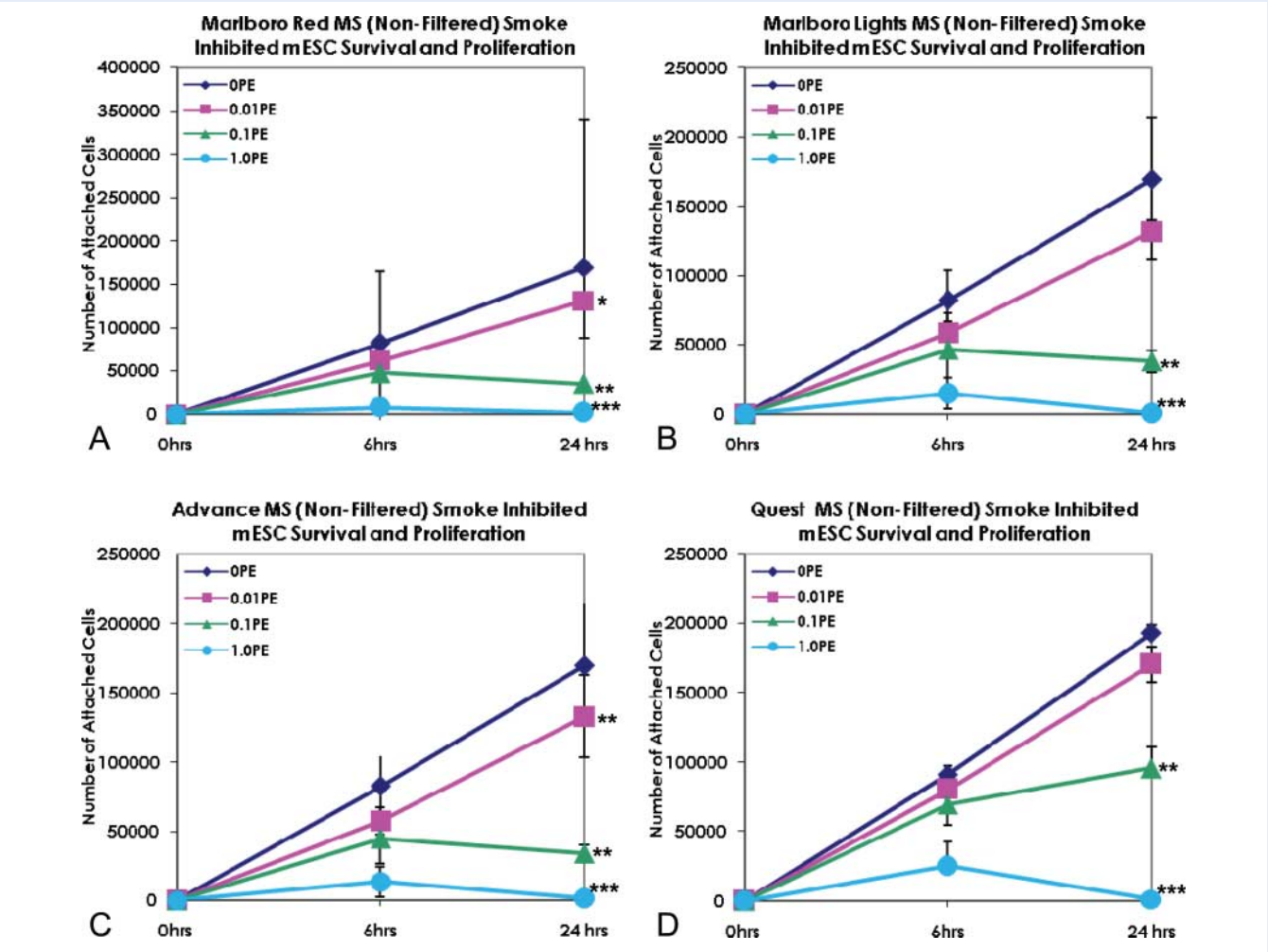

Figure 5 Non-filtered MS smoke inhibited mESC survival and proliferation dose dependently.
(**A**) Marlboro Red non-filtered MS smoke, (**B**) Marlboro Lights non-filtered MS smoke, (**C**) Advance Lights non-filtered MS smoke and (**D**) Quest non-filtered MS smoke. Each point is the mean ± SD of 3 experiments. Statistical significance was determined using a one-sample *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

smoke treatment. Pre-attached cells underwent detachment upon exposure to either MS or SS smoke. The magnitude of this effect was very similar in terms of potency whether cells were attached first or treated with smoke during attachment (compare Figs 4 and 6 with Fig. 7). The attachment of human ESC to Matrigel is inhibited by nicotine, a major constituent of tobacco smoke (Zdravkovic *et al*., 2008). In our studies, Quest, which does not contain nicotine, was effective at inhibiting attachment of mESC to gelatin suggesting that in complex mixtures of smoke more than one molecule is involved in blocking attachment. Similar interference of smoke with attachment of both undifferentiated and differentiated cells has been observed in other systems. In humans, cigarette smoking down regulates the l-selectin adhesion system in the placenta, an effect that appears to be mediated by nicotine (Zdravkovic *et al*., 2006). Similarly, differentiated bovine bronchial epithelial cell attachment to fibronectin *in vitro* is inhibited by short-term exposure to cigarette smoke condensate (Cantral *et al*., 1995).

Proliferation and growth are among the most important processes in prenatal development. At no other time does mitosis occur at such a high rate in mammals. Both MS and SS smoke solutions from traditional and harm-reduction cigarettes inhibited proliferation (lower doses) and survival (higher doses) of mESC. At high doses, apoptosis was induced through caspase activation. If toxicants in smoke similarly decrease proliferation and/or survival of cells in preimplantation embryos of human smokers, the consequences could be very significant. Possible outcomes could include reduced birthweight, as has been reported in offspring of women who smoke (Andres and Day, 2000; Hruba and Kachlik, 2000; Steyn *et al*., 2006), reduced populations of stem and progenitor cells that would be necessary for subsequent growth and organ development, or development of congenital defects due to loss of normal cell numbers. Defects due to increased apoptosis have been observed in explanted mouse embryos cultured in the presence of nicotine (Zhao and Reece, 2005), and digit defects have been reported to increase in offspring of women who smoke (Man and Chang, 2006).

It is interesting that smoke treatment of mESC, in most cases, did not kill all mESC. In most treatment groups, even at the highest doses, some cells survived, and at lower doses, even proliferated,

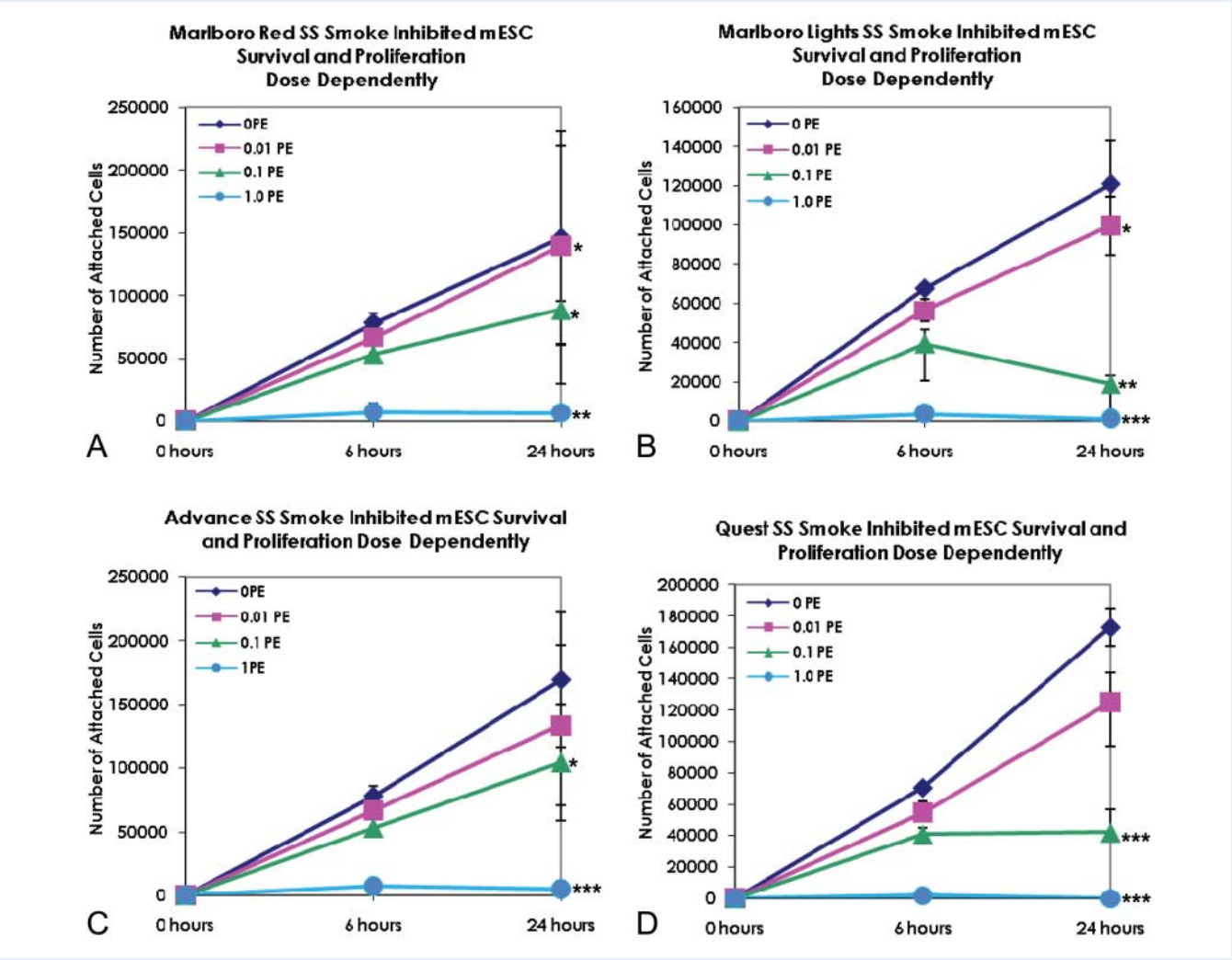

Figure 6 SS smoke inhibited mESC survival and proliferation dose dependently.
(**A**) Marlboro Red SS smoke, (**B**) Marlboro Lights SS smoke, (**C**) Advance Lights SS smoke and (**D**) Quest SS smoke. Each point is the mean ± SD of three experiments. Statistical significance was determined using a one-sample *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

albeit at slower rates than the control. Therefore smoke exposure may not outright kill all cells in preimplantation embryos, but may reduce cell numbers by inhibiting division or killing a fraction of the cell population. Indeed, in the actual preimplantation mouse embryos that we examined, not all blastomeres were killed by smoke treatment. This interesting result suggests that there is a population of cells within the mESC population, and by extension within the inner cell mass the preimplantation embryo, that is more sensitive to smoke treatment than the surviving cells. It would be interesting to know if the cells that are killed are in some way developmentally distinct from those that survive and grow normally.

When mESC were pretreated for only 1 h with smoke solutions, they likewise were inhibited from attaching and as seen in the prior experiments, survival of those that attached was impaired. The magnitude of these effects was greater for SS smoke than for MS smoke. These data indicate that the action of smoke on the mESC occurs relatively quickly, and in this experimental design was not readily reversible. If similar effects occur in women who smoke, damage could be done to the preimplantation embryo well before the smoker knew that she was pregnant.

Our data revealed several important points about the types of cigarette smoke that were tested with mESC. As we have reported previously using other assays (Knoll and Talbot, 1998; Melkonian *et al.*, 2000; Melkonian *et al.*, 2002; Gieseke and Talbot, 2005; Riveles *et al.*, 2007), SS smoke from all brands of cigarettes was consistently more toxic than MS smoke in the mESC assays. SS smoke contains higher levels of potential toxicants than MS smoke which is produced by burning tobacco at a higher temperature (EPA, 1992; Riveles *et al.*, 2007). In our study, SS smoke solutions were compared with MS smoke on a PE basis. In an actual smoking situation, SS smoke would be diluted in air before being inhaled by an active or passive smoker, and this dilution would tend to benefit the smoker. However, levels of SS smoke in some passive smoking environments can be high (e.g. in an enclosed automobile or smoky bar) and exposure may occur for relatively long periods of time. Our data indicated that such exposures may not be safe for pregnant women with preimplantation embryos.

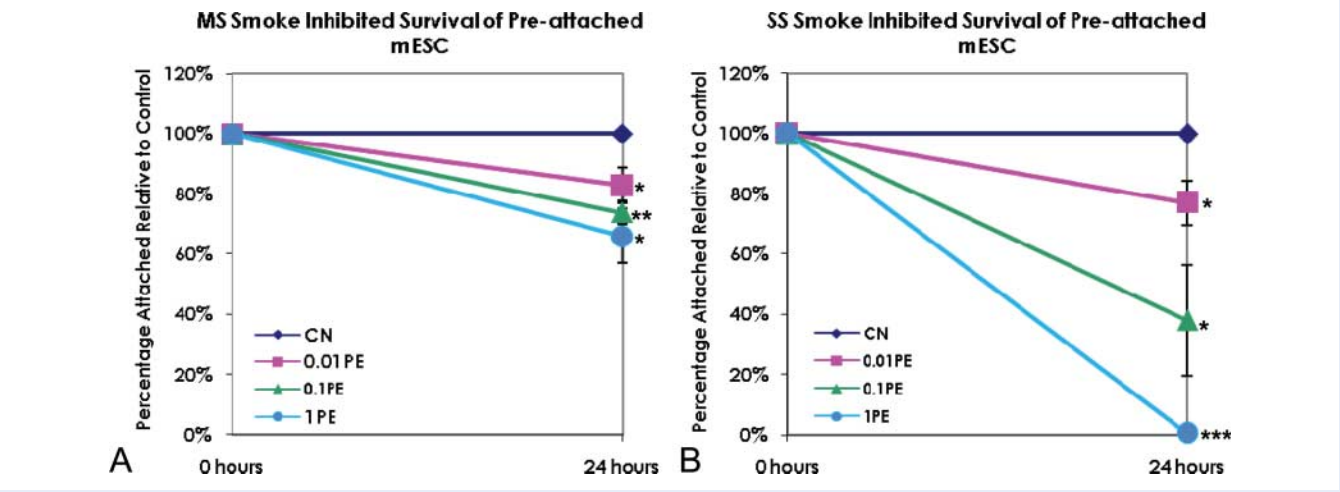

Figure 7 Advance MS (filtered) and SS smoke solutions caused detachment of pre-attached mESC dose dependently.
Each point is the mean ± SD of three experiments. Statistical significance was determined using a one-sample *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

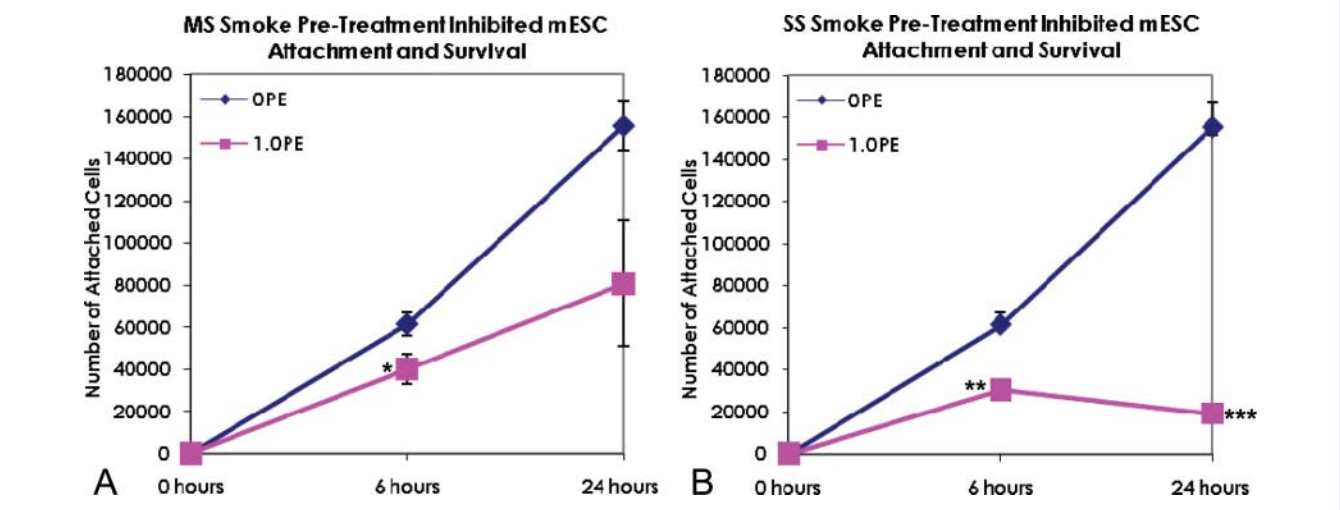

Figure 8 Pretreatment of mESCs with Advance MS (filtered) (**A**) or SS (**B**) smoke solutions before plating inhibited attachment (MS and SS), survival (SS) and proliferation (MS).
Each point is the mean ± SD of three experiments. Statistical significance was determined using a *t*-test at 6 h and at 24 h (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

When comparing filtered and non-filtered MS smoke, the presence of a filter on the cigarette greatly reduced the potency of MS smoke, although the filter was not sufficient to completely eliminate toxicity. While filters are generally thought to remove toxicants, our data specifically showed that mESC and by extension preimplantation embryos benefit by filtering MS smoke before exposure to it. Since SS smoke cannot be filtered, there is not a clear simple way to reduce the toxicants released into the environment from this type of smoke. Moreover, active smokers who use filtered cigarettes will also be exposed to their own SS smoke which, in combination with their inhaled MS smoke, may pose a threat to preimplantation embryos.

Our data show that, in the mESC assay, MS and SS smoke solutions from harm-reduction cigarettes are as potent or more potent than smoke from a traditional brand (Marlboro Red). We previously showed that smoke from harm-reduction cigarettes impaired functioning of the hamster oviduct in ciliary beat frequency, oocyte collection rate and muscle contraction assays (Riveles *et al*., 2007). In general, in both studies, SS smoke was more potent than MS smoke. However, the mESC were overall more sensitive to lower doses of harm-reduction smoke than the oviduct, supporting the idea that developing tissues are more severely affected by environmental toxicants than adult tissues (Grandjean *et al*., 2007), and demonstrating the importance of assessing toxicity on prenatal stages of development. Harm-

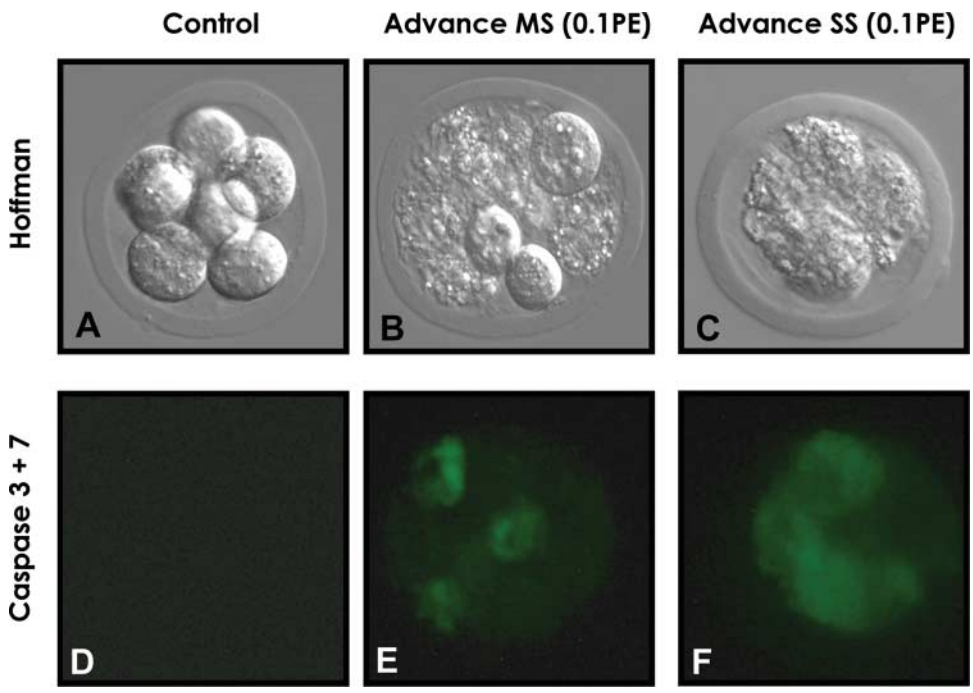

Figure 9 Smoke treatment induced apoptosis in mouse preimplantation embryos through activation of caspases 3 and 7.
(**A**–**C**) Hoffman contrast images of preimplantation embryos incubated in control medium (A), 0.1 puff equivalents (PE) of Advance MS smoke solution (B), or 0.1PE of Advance SS smoke solution (C). (**D**–**F**) The same preimplantation embryos viewed with fluorescence microscopy to visualize activated caspases 3 and 7. (D) Absence of activated caspases 3 and 7 in blastomeres of control untreated embryo, (E) Presence of activated caspases 3 and 7 in embryo treated with Advance filtered MS smoke, (F) Detection of activated caspases 3 and 7 in embryo treated with SS smoke.

reduction cigarettes are marketed as having lower levels of carcinogens and accordingly being safer to smoke (Warner, 2005). This can give smokers a false sense of security as they think that harm-reduction brands lower their exposure to toxicants (Shiffman *et al.*, 2001; Hamilton *et al.*, 2004; Pederson and Nelson, 2007). However, it has never been shown that harm is in fact reduced when using these products (Pankow *et al.*, 2007), and our tests with harm-reduction smoke clearly showed this type of smoke retains toxicity. In the mESC assays, toxicants that could affect prenatal development were still present in filtered MS smoke from harm-reduction brands, while SS smoke, which is not filterable, was highly toxic.

Finally, embryos and fetuses are generally more sensitive to toxicants than adults, and it is important to adjust acceptable levels of exposure to toxicants to levels that are not damaging *in utero* (Grandjean *et al.*, 2007). New *in vitro* assays are needed that can be used to screen the effects of environmental chemicals on various stages of prenatal development. It is particularly important to have assays that monitor different prenatal stages since time of exposure to toxicants is as important as dose. Other assays have been developed to monitor later stages in development using explants of rodent embryos (e.g. Joschko *et al.*, 1991; Zhao and Reece, 2005) and using ESC formed in to embryoid bodies that model post-implantation development (Seiler *et al.*, 2006). The assay that we used in this study provides a simple, quick method for assessing environmental toxicants on mESC, a model for the inner cell mass of preimplantation embryos. This assay could be used to screen any chemical for toxicity on this stage of development. Given the high percentage of embryos that never implant or that spontaneously abort within two weeks of implantation, it is likely that the preimplantation stages of development, which occur before a woman knows that she is pregnant, are very critical and important to study. We are currently adapting our assay to human ESC which will give more direct information on our species.

In conclusion, mESC provide a rapid assay for modeling the effects of environmental toxicants, such as tobacco smoke, on preimplantation stages of development. By using mESC, animal usage can be minimized, and data can be obtained on important developmental parameters within a 24 h period. Moreover, the murine model provides easy access to resources, a rapid straightforward assay, and minimizes ethical controversies. This study confirms the effectiveness of using ESC as a novel model to study embryo toxicity during preimplantation development. Further work is being directed at developing human ESC for similar testing. Using mESC to evaluate cigarette smoke enabled relative comparisons between the toxicity of different types of smoke from different brands of cigarettes. Our data confirm that on a per puff basis, SS smoke is more toxic than MS smoke and that smoke from harm -reduction cigarettes is as potent or more potent than smoke from a traditional brand.

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