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# In Vitro Assessment of Reproductive Toxicity of Tobacco Smoke and Its Constituents

Prudence Talbot

Epidemiological studies have repeatedly shown that reproductive processes in pregnant women are adversely affected by exposure to cigarette smoke. The potential reproductive targets of smoke during pregnancy include the ovaries, oviducts, uterus, placenta, umbilical cord, and embryo/fetus. In vitro methods for studying the effects of smoke and its individual components have been developed and applied to each of these reproductive targets. In vitro assays have been useful in determining the biological processes that are affected in the reproductive organs and in identifying the cellular and molecular targets of smoke in each organ. In vitro methods have also been used to study the mechanism of action of smoke constituents, such as nicotine, on specific processes in reproductive organs and to screen smoke solutions to identify the molecules that affect reproduction. In general, data collected in vitro have confirmed, extended, and helped explain what has been learned from epidemiological studies. This review summarizes some of the in vitro assays that have been used to study cigarette smoke's effect on the nonpregnant and pregnant female reproductive tract and spotlights examples of their applications. **Birth Defects Research (Part C) 84:61–72, 2008.** © 2008 Wiley-Liss, Inc.

**Key words:** reproductive toxicity; invitro assays; cigarette smoke; tobacco

## INTRODUCTION Pregnancy—Is it Affected by Cigarette Smoke Exposure?

The adverse effects of cigarette smoke on human female reproductive health have been demonstrated in numerous epidemiological studies that correlate both active and passive smoking with increased risks for developing reproductive disorders (Stillman et al., 1986; Augood et al., 1998; Shiverick and Salafia, 1999; Higgins, 2002; Ashmead, 2003). Active and passive smoking significantly reduce birth weight (Windham et al., 1999; Hrubá and Kachlik, 2000; Berthiller and Sasco, 2005), while active smoking also increases placental abruption, premature delivery, spontaneous abortion, perina-

tal mortality, and ectopic pregnancy (Ahlborg and Bodin, 1991; Andres and Day, 2000; Higgins, 2002; Ford and Dobson, 2004; Jauniaux and Burton, 2007). More recently, it has been recognized that fetal exposure to smoke can increase postnatal morbidity and mortality and can lead to impaired cognitive and behavioral development (DiFranza et al., 2004; Perera et al., 2006).

In addition to epidemiological data, much of our knowledge regarding smoke's effect on human health comes from studies done using in vitro models. The purpose of this review is to summarize examples of the in vitro methods that have been used to study the effect of smoke on reproductive

targets in pregnant and nonpregnant females and to spotlight some of the data obtained with these methods.

## Why Use In Vitro Models and Assays to Study Tobacco Smoke's Effect on Reproduction?

We do not fully understand smoke's effects on human reproduction or the mechanisms by which smoke exerts its effects. Both in vitro and in vivo models can help increase our understanding, however, some reproductive processes are difficult to study in humans or animal models. In vitro assays offer a way to test cigarette smoke and its constituents in a controlled experimental environment. In vitro models have been invaluable in helping identify reproductive targets of smoke and the biological effects exerted by smoke and its individual components. The use of in vitro models has helped clarify what smoke does to reproductive processes, how it exerts its negative effects, and which specific chemicals in smoke are harmful. In vitro models enable manipulation of experimental designs to establish effective doses and biological outcomes. In vitro assays also permit rapid screening of specific chemicals in smoke for toxicity and risk assessment, and identified toxicants can then be studied in more expensive and challenging in vivo experiments. Whenever possible, in vivo

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**Figure 1.** Example of a smoking machine that is used to create smoke solutions for in vitro testing. A peristaltic pump attached to a puffer box draws a 2 second 35 ml puff of mainstream smoke every minute. The smoke is passed through culture medium (tube to medium is not connected). The machine can also be used to make sidestream smoke solutions.

experiments are important for confirming in vitro data.

Many in vitro assays that measure cytotoxicity and genotoxicity have been used to evaluate tobacco smoke, and much of this literature has recently been reviewed (Andreoli et al., 2003). In this review, we will focus on in vitro methods that measure biological activity or functioning of reproductive targets in pregnant and non-pregnant females.

### Testing Tobacco Products In Vitro

Delivery of smoke to an in vitro model is usually accomplished by collecting smoke on a cold glass surface or filter then extracting and testing the condensate (Anto et al., 2002). Solutions of tobacco smoke can also be made by drawing smoke through culture medium and testing the resulting solution at various doses (Knoll and Talbot, 1998). When working with solutions, whole smoke can be tested, or the particulate phase (collected on a filter) and gas phase (portion that passes through the filter) can be collected and tested separately (Melkonian et al., 2002). In some studies, smoke has been drawn directly over cultured cells or culture medium has been exposed to

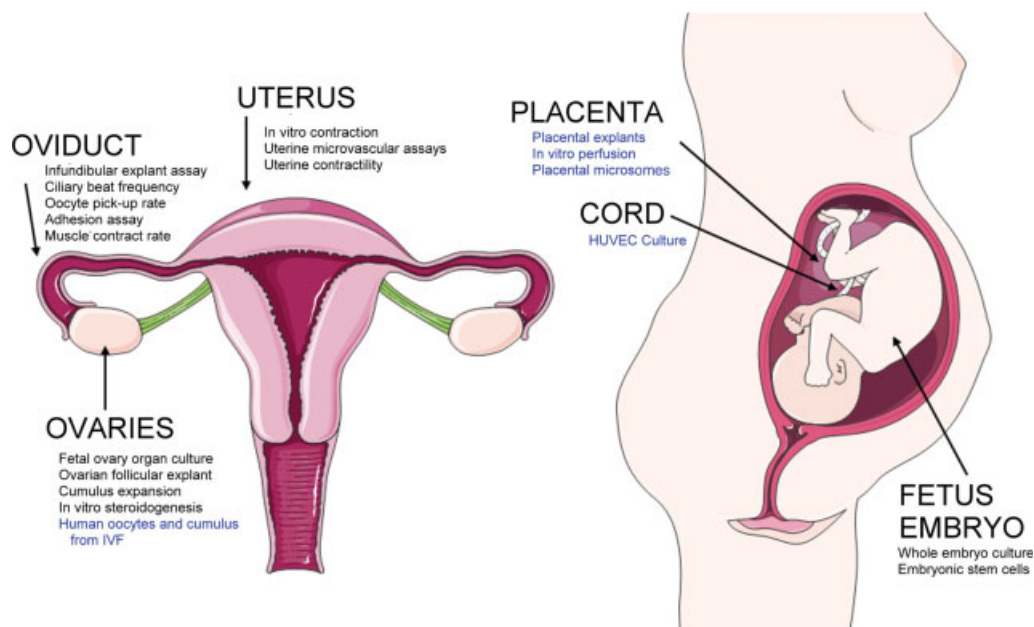
smoke for variable lengths of time to produce smoke conditioned medium (Soghomonians et al., 2004; Vidal et al., 2006). When making smoke solutions and condensates, cigarettes are usually smoked using a protocol established by the Federal Trade Commission/International Organization for Standardization (FTC/ISO) (Group, 2007) which involves a 35 ml puff of 2 sec duration every minute (Fig. 1). However, because it is now realized that smokers often adjust their inhalation to compensate for low tar and low nicotine cigarettes, other smoking machine protocols have been developed. The Health Canada protocol is based on 55 ml puff volume, 30 sec puff interval, 2 sec puff duration, and ventilation holes fully blocked to more accurately simulate heavy smoking. Interestingly, smokers who use "light" cigarettes compensate for low nicotine by inhaling deeper or smoking more cigarettes and receive higher doses of toxicants than those smoking traditional cigarettes with standard inhalation (Benowitz et al., 1983; Djordjevic et al., 1997). Thus, one caveat of any type of in vitro exposure is knowing how to mimic human smoking. In fact, this is virtually impossible to do since inhalation parameters vary among humans

and are affected by the level of nicotine in the product. However, the advantage of using a smoking machine to create solutions and condensates for in vitro testing is that the production of the test solutions can be accurately and precisely controlled. In addition, the amounts of key chemicals, such as nicotine or polycyclic aromatic hydrocarbons (PAHs), in smoke solutions and condensates can be measured, which enables in vitro experiments to be done using quantified exposures. Any data obtained in vitro must, however, be viewed with the realization that not all smokers inhale cigarette smoke the same way and that specific individuals may be more or less affected than in vitro dose data suggest. Moreover, when possible, any effect observed in vitro needs to be confirmed in vivo since toxicants in smoke may be metabolized to a nontoxic form or activated to a harmful form in vivo.

### Tobacco Products and Tobacco Smoke

Various types of tobacco products are available to women. These include traditional cigarettes, harm reduction cigarettes, cigars, and smokeless tobacco products, such as snus. Traditional cigarettes are the product most commonly used by women and likewise the most studied. In the United States, approximately 30% of reproductive-aged women smoke traditional cigarettes. This percentage varies considerably in other countries, but is rising worldwide. Therefore, of the various types of tobacco exposure possible, the effects of smoke from traditional cigarettes are of most concern to human reproduction and pregnancy outcomes.

Cigarette smoke is a complex colloid containing more than 4,000 chemicals. Some of these chemicals originate in the cigarette itself, while others are produced during burning or are added to the cigarette during manufacture to improve flavor. Many of these chemicals are known toxicants, and nicotine, a major constituent of tobacco smoke, causes addiction (Alouf et al., 2006). However, most of the chemicals in smoke



**Figure 2.** Targets of cigarette smoke in nonpregnant and pregnant females. In vitro data support the idea that the ovaries, oviducts (Fallopian tubes), and uterus are targets of smoke in the non-pregnant female. During pregnancy additional targets include the placenta, umbilical cord, and embryo/fetus. In vitro assays that have been used with each target are shown. Blue text indicates assays done with human cells.

have not yet been analyzed for their toxicological properties, and smoke probably contains many toxicants that are as yet unidentified. Methods that enable screening for toxicity are needed, and in vitro approaches have proven useful for this purpose in the past (Talbot and Riveles, 2005).

Burning a cigarette produces two major classes of smoke. Mainstream smoke is the puff or bolus of smoke inhaled by an active smoker, while sidestream smoke burns off the end of a cigarette and is the main component of environmental tobacco smoke (also called second-hand smoke). In addition to sidestream smoke, environmental tobacco smoke contains the smoke that an active smoker exhales. Sidestream smoke is inhaled by both active and passive smokers. The chemical composition of both types of smoke is similar; however, the concentration of individual components varies in each type, and some toxicants are considerably higher in concentration in fresh sidestream smoke (EPA, 1992). This difference in concentrations is apparently due to the different temperatures at which tobacco burns

when producing mainstream and sidestream smoke. Also mainstream smoke is sometimes filtered whereas sidestream smoke is not filtered. While toxicants' concentrations in sidestream smoke tend to be higher than their counterparts in mainstream smoke, the concentration of sidestream smoke inhaled by a smoker is quite variable and depends on the degree of its dilution in air before inhalation occurs. Tobacco can deliver nicotine in various products that are chewed, not smoked, such as snuff and snus (Hatsukami et al., 2004). In general, however, these are not used by pregnant women, although their use has been advocated as a product that may be somewhat less dangerous than traditional cigarettes (Rodu and Godshall, 2006).

### Reproductive Targets of Cigarette Smoke in Females

In pregnant women, the potential reproductive targets of cigarette smoke are the ovaries, oviducts, uterus, placenta, umbilical cord, and embryo/fetus (Fig. 2). Each of these targets has been the subject of in vitro testing. In some

cases, such as the placenta, it is relatively easy to obtain human tissue for in vitro testing. In fact, some studies on placenta have successfully correlated in vivo exposure of pregnant women to smoke with in vitro experimental data obtained using their placentas (Genbacev et al., 2000). Human umbilical vein endothelial cells (HUVEC) also represent an in vitro model that uses human cells and has been used frequently in smoke-related studies. Some in vitro models have relied on tissue from other species which in general have included the mouse, rat, hamster, cow, or pig.

### In vitro Assays that Have Been Used in Tobacco-Related Research on Reproduction

#### *Ovaries and oocytes*

Mammalian ovaries appear to have a finite number of oocytes at birth (Mandl and Zuckerman, 1951), although this concept has been challenged recently (Johnson et al., 2004, 2005). The limited number of oocytes in women makes the toxicity of smoke to the ovary very important, as ovotoxicants in smoke potentially reduce fertility of women

by accelerating oocytes loss (Mattison et al., 1989). Women who smoke enter menopause earlier than do nonsmokers; perhaps, because of antiestrogenic effects of smoke accompanied by oocyte loss (Tanko and Christiansen, 2004). Processes within the female reproductive tract are controlled hormonally both with gonadotropins originating in the anterior pituitary gland and with steroid hormones originating in the ovary. Anything that upsets the balance of the hormonal interactions within the tract can impair reproduction.

**In vitro fetal ovary organ culture.** The effects of smoke and its constituents on oocytes have been studied extensively both in vivo and in vitro (Mattison et al., 1989; Mlynarcikova et al., 2005). Well developed methods for culturing ovarian follicles and oocytes make the ovary particularly amenable to in vitro studies (Hartshorne, 1997), and recent methods for culturing fetal ovaries have extended in vitro studies to developing ovaries.

The fetal ovarian organ culture system involves removing genital ridges from female fetuses on embryonic day 13.5 and culturing them in vitro using conditions that minimize apoptosis in controls (Morita et al., 1999). After treatment with the test compounds, ovaries are fixed, sectioned, and the number of oocytes or follicles is counted to determine if treatment reduced their number. While the use of in vitro assays is very powerful in this type of experiment, one difficulty has been accurately counting oocytes in ovaries (Tilly, 2003); however, newer validated methods are improving the accuracy of these counts (Myers et al., 2004).

Fetal ovarian organ culture was used to study the mechanism of action of PAHs on oocyte survival (Matikainen et al., 2002). It has been known for many years that cigarette smoke contains ovotoxicants including PAHs that when injected into mice cause loss of young oocytes (Mattison et al., 1989). Moreover, treatment of pregnant mice with PAHs or cigarette smoke reduces the number of

oocytes in their female offspring (MacKenzie and Angevine, 1981; Vahakangas et al., 1985). Using the fetal ovarian culture system, experimental groups were treated with a PAH called DMBD-3-4-dihydrodiol (DMBA-DHD), which caused a dose dependent decrease in oocyte number that was reversible by the aromatic hydrocarbon receptor (AHR) antagonist  $\alpha$ -naphthoflavone (ANF). DMBA-DHD treated ovaries showed a marked increase in immunoreactivity of Bax, a proapoptotic factor, which could be eliminated by ANF. An in vivo follow-up study showed that Bax mutant fetuses exposed to PAHs were born with a normal number of oocytes in contrast to wild-type controls which had Bax and lost oocytes in response to PAH treatment. The use of the in vitro ovarian organ culture helped establish that DMBA-DHD binds to the AHR which in turn activates Bax leading to loss of fetal oocytes.

#### **Ovarian follicular explants.**

Rat ovarian follicles have been explanted and cultured in vitro (isolated rat follicle culture) (Neal et al., 2007). This assay involves isolating 80 to 100  $\mu$ m diameter follicles from ovaries, treating them in vitro with test compounds, and measuring the effect on follicle growth. This assay was used to show that benzo-[a]-pyrene, a PAH in cigarette smoke, inhibits FSH stimulated follicular growth dose dependently (Neal et al., 2007). In this study, the concentrations of benzo-[a]-pyrene were also measured in the serum and follicular fluid of smoking and non-smoking women undergoing in vitro fertilization procedures. Interestingly, benzo-[a]-pyrene levels were significantly higher in follicular fluid than in serum of smokers, and the mean concentration (1.5 ng/ml) present in smokers' follicular fluid significantly inhibited in vitro follicular growth in the rat model. This assay provides another in vitro method for studying toxicity of smoke on follicles and their oocytes.

#### **In vitro cumulus expansion.**

Normally in tertiary or Graafian

ovarian follicles, cumulus cells surrounding the oocyte undergo expansion in response to luteinizing hormone before ovulation (Phillips and Dekel, 1991). Expansion is brought about by secretion of hyaluronic acid which combines with extracellular proteins to form a stable matrix between cumulus cells (Chen et al., 1996). Cumulus expansion normally occurs in ovarian follicles before ovulation of the oocyte cumulus complex (OCC) and appears to be necessary for successful ovulation (Talbot, 1983) and pick-up of oocytes by the oviduct (Talbot et al., 2000).

The effect of cigarette smoke on cumulus expansion has been studied in vitro using FSH induced expansion in the porcine model (Vrsanska et al., 2003; Mlynarcikova et al., 2004). In this assay, porcine OCC was isolated from 5 to 8 mm follicles and cultured in vitro in varying doses of cadmium, anabasine, or nicotine, all components of cigarette smoke. The end point for this assay is the degree of expansion, which was evaluated using a subjective scoring index. Treated OCC failed to expand as much as untreated controls, and hyaluronic acid synthesis, a prerequisite for successful expansion, was significantly reduced by treatment (Vrsanska et al., 2003). These data show that specific chemicals in smoke inhibit OCC expansion which could interfere with ovulation and transfer of the OCC to the oviduct.

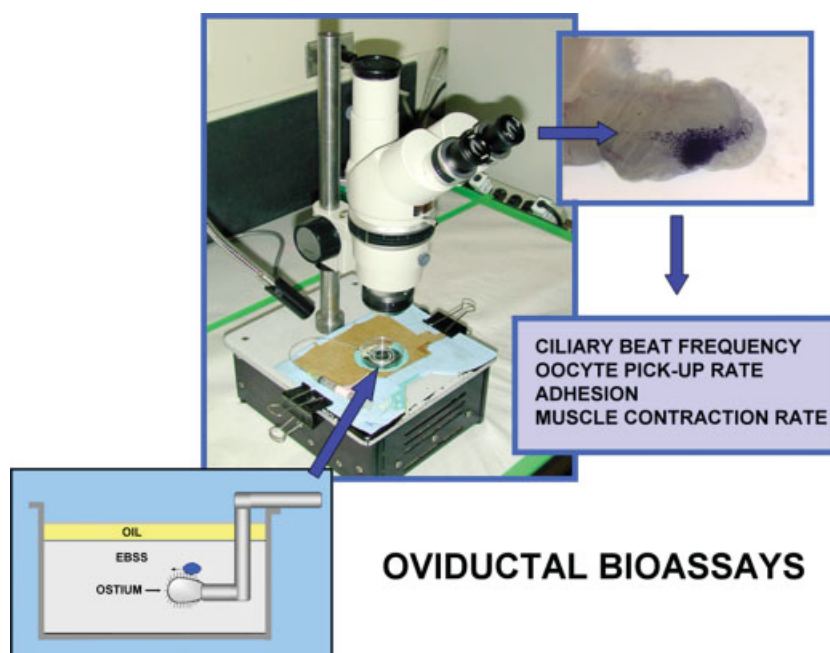
**In vitro steroidogenesis.** In vitro models have also been used to assay the effect of smoke components on steroid production of both human and bovine ovarian follicles. The human model uses granulosa cells harvested from patients undergoing in vitro fertilization. Exposure of cultured human granulosa cells to smoke or nicotine has given mixed results with respect to estradiol synthesis. In some studies (Barbieri et al., 1986b; Vidal et al., 2006), inhibition of estradiol synthesis was reported following treatment with mainstream smoke, environmental tobacco smoke,

or nicotine, but similar inhibition was not found by others (Bodis et al., 1997; Gocze et al., 1999; Weiss and Eckert, 1989). In a recent study, human luteinized granulosa cells were exposed to medium conditioned by environmental tobacco smoke and showed a decrease in both estrogen and progesterone production (Vidal et al., 2006). In a subsequent study using the bovine model, both theca interna and granulosa cells were cultured in vitro and treated with nicotine or cotinine (Sanders et al., 2002). These authors found inhibition of androstereone secretion (a precursor of estradiol) by theca interna cells treated with nicotine but not with cotinine. However, none of the chemicals significantly affected estradiol secretion by granulosa cells. Further information on steroidogenesis has recently been reviewed (Mlynarcikova et al., 2005).

**In vitro assessment of oocytes and cumulus cells from in vitro fertilization patients.** In vitro fertilization (IVF) laboratories have provided human oocytes for in vitro assessment of smoke's effects on oocyte quality and fertilizability. In these studies, the material used comes from documented smokers or nonsmokers and evaluations are done in vitro following removal from the patient. This approach showed that oocyte maturation was inhibited in smokers, who produced significantly more diploid oocytes than did nonsmokers (Zenzes et al., 1995). In addition, cumulus cells collected during IVF procedures showed more DNA damage in smokers than did nonsmokers (Sinko et al., 2005). Zona pellucida thickness has also been found to be thicker around oocytes from active and passive smokers (Shiloh et al., 2004).

### Oviducts

The mammalian oviduct is a multifunctional organ that is anatomically divided into the infundibulum (closest to the ovary), ampulla, and the isthmus (closest to the uterus) (Harper, 1994; Talbot and Riveles, 2005). The epithelial lining



**Figure 3.** The infundibular explant assay. An infundibulum from a hamster oviduct is mounted in a holding pipette and test solution is placed on the explant. Four parameters can be monitored before, during, or after treatment. These include ciliary beat frequency, OCC pick-up rate, adhesion of the OCC to the oviduct, and muscle contraction rate.

of the oviduct is both ciliated and secretory throughout its length with cilia being more prevalent in the infundibulum and gradually becoming fewer towards the isthmus. The wall of the oviduct contains smooth muscle that is particularly abundant in the isthmus. Pick-up of the OCC by the oviduct depends on adhesion of the cumulus matrix to the tips of the cilia and beating of the cilia toward the ostium (opening of the infundibulum) (Norwood and Anderson, 1980; Mahi-Brown and Yanagimachi, 1983; Talbot et al., 2000). The oocyte enters the oviduct rapidly after ovulation then slows its movement in the ampulla where fertilization occurs. Sperm are transported in the opposite direction of the oocyte, towards the site of fertilization (Blandau and Verdugo, 1976; Suarez, 2002). The fluid milieu of the oviduct provides an environment suitable for fertilization and preimplantation development and the oviduct through ciliary beating and smooth muscle contraction conveys the preimplantation embryo to the uterus

where it implants in the endometrium.

### Infundibular explant assay.

While entire oviducts are difficult to culture for long periods of time in vitro, explants of the hamster infundibulum have been successfully cultured for short-term experiments and used to monitor the effects of tobacco smoke and its constituents on oviductal processes (Huang et al., 1997; Knoll and Talbot, 1998; Riveles et al., 2003). Because the oviduct is multifunctional, separate assays have been developed for evaluating ciliary beat frequency, adhesion of the OCC to the cilia of the oviduct, oocyte pick-up rate, and smooth muscle contraction rate in infundibular explants. The assays involve removing the infundibulum from the hamster oviduct and culturing it in vitro in a dish fitted with a capillary holding pipette. The small piece of the ampulla is mounted in the pipette by suction leaving the infundibulum exposed for experimentation (Fig. 3). The infundibular explants can be applied

to any type of biological testing including evaluation of smoke.

**Infundibular assay—Ciliary beat frequency.** Ciliary beat frequency is of interest in smoke-related studies because cilia clear mucus from the respiratory tract, which is the first site of smoke exposure in humans, as well as move OCC into and through the oviduct. Cigarette smoke contains chemicals that inhibit ciliary beating in a variety of mammalian and non-mammalian models (Wang, 1963; Dalhamm, 1970; Battista, 1974; Zayas et al., 2004). Ciliary beat frequency has been measured from video recordings using the hamster infundibular bioassay before, during, and after exposure to solutions containing whole smoke, particulate smoke, or the gas phase of smoke (Knoll et al., 1995). Whole mainstream smoke and its fractions significantly inhibited ciliary beating, which partially recovered after washout (Knoll et al., 1995), while whole sidestream smoke solutions slightly stimulated beat frequency (Knoll and Talbot, 1998). The decrease in beat frequency seen with mainstream smoke treatment could retard the rate of oocyte pick-up or even prevent pick-up from occurring, and could be a factor in the increased incidence of ectopic pregnancy seen in women who smoke.

**Infundibular assay—Oocyte pick-up rate.** The infundibular explant can be used to measure oocyte pick-up rate, one of the most important functions of the oviduct (Huang et al., 1997; Knoll and Talbot, 1998). This assay is done by placing an OCC on the surface of the infundibulum away from the ostium (opening into the oviduct) and measuring the time required for the OCC to move to the ostium (Huang et al., 1997). OCC pick-up rate was inhibited dose dependently by both mainstream and sidestream smoke solutions, and at equivalent doses, undiluted sidestream smoke was more potent than mainstream smoke (Knoll and Talbot, 1998). Interestingly, sidestream smoke inhibited OCC pick-up rate even

when ciliary beat frequency was not inhibited or was slightly stimulated (Knoll and Talbot, 1998), demonstrating that factors in addition to ciliary beating were important in pick-up. Smoke from harm reduction cigarettes, which have lower levels of carcinogens than traditional brands, was likewise inhibitory in this assay (Riveles et al., 2007), and mainstream smoke from one harm reduction brand was more inhibitory than smoke from any traditional or research brand tested.

**Infundibular assay—Adhesion assay.** Small objects such as *Lycopodium* spores can be picked up by ciliary currents and moved directly into the oviduct (Gaddum-Rosse and Blandau, 1976; Talbot et al., 1999). However, the mass of the OCC is large relative to the cilia, and currents do not sweep OCC through the ostium. Rather OCCs adhere to the infundibular surface, and ciliary beating "walks" the OCC over the surface and through the ostium (Talbot et al., 1999). Adhesion occurs between small electron dense crowns on the tips of the oviductal cilia and the extracellular matrix between cumulus cells in the OCC (Lam et al., 2000). In scanning electron micrographs, strands of the OCC matrix can be observed on the surface of the infundibulum after an OCC has passed over it.

Since adhesion plays a crucial role in OCC pick-up, the oviductal explant assay was developed to measure adhesion between the tips of the cilia and the OCC (Lam et al., 2000; Gieseke and Talbot, 2005). This assay uses a small peristaltic pump that can be calibrated to measure the flow rate required to suction an OCC from the surface of the infundibulum and provides a quantitative measure of adhesion.

Relative to untreated controls, whole mainstream smoke solutions and their particulate and gas phase fractions inhibited OCC pick-up rate and increased adhesion when either the infundibulum or OCC was pretreated with smoke solution, although a stronger inhibition occurred with infundibular pre-

treatment (Gieseke and Talbot, 2005). Sidestream smoke produced a similar effect which was isolated to the gas phase. The sidestream smoke data also showed that even when ciliary beat frequency was normal, inhibition of OCC pick-up occurred because of increased adhesion. These data demonstrate the importance of adhesion in the pick-up process and further show that both the oviduct and OCC are targets of smoke. If a similar phenomenon occurs in humans, then the OCC may not be picked up at all in smokers in which case fertilization could occur outside of the oviduct which may lead to peritoneal implantation. Alternatively, the OCC may get picked up but move too slowly through the oviduct which could also lead to ectopic implantation in the oviduct.

**Infundibular assay—Contraction of oviductal muscle.** Oviductal smooth muscle plays a role in movement of fertilized oocytes and preimplantation embryos through the oviduct (Croxatto, 2002). Since prior in vivo studies had shown inhibition of oviductal smooth muscle contraction is correlated with decreased movement of preimplantation embryos through the oviduct (DiCarantonio and Talbot, 1999), the infundibular assay was further developed to measure contraction of infundibular muscle in vitro (Riveles et al., 2003). Strength of contraction was determined by measuring the distance the infundibulum moves during a contraction. Frequency was determined by counting the number of contractions per minute.

When infundibula were treated with mainstream or sidestream smoke solutions from traditional commercial cigarettes, both types of smoke from all but one brand significantly inhibited oviductal muscle contraction. Always, where inhibition occurred, sidestream smoke was more potent than mainstream (Riveles et al., 2007). When three harm reduction brands were tested, all inhibited muscle contraction, except mainstream smoke from one brand. Again sidestream smoke was more potent, and reduced

contraction rates to 30% or less of the control rate for three harm reduction brands tested. These data show that chemicals in cigarette smoke inhibit oviductal contractions, in agreement with *in vivo* data (DiCarlantonio and Talbot, 1999), and help explain why embryo transport is retarded in females inhaling smoke. If similar inhibition of oviductal contraction occurs in human smokers, the effects could again lead to ectopic implantation in the oviduct or infertility; both problems seen in women smokers.

**Infundibular assay—Use in chemical genomics.** The *in vitro* infundibular assay was used to test five chemicals previously reported to be ciliotoxic in other models. While all chemicals (potassium cyanide, formaldehyde, acetaldehyde, acrolein, and phenol) inhibited oviductal beat frequency dose dependently, only potassium cyanide was present in smoke solutions in high enough concentration to account for this inhibition (Talbot et al., 1998). Since it was probable that smoke contained additional oviductal toxicants, the *in vitro* infundibular assay was used to bulk screen solutions of cigarette smoke and identify the chemicals that inhibit beat frequency, OCC pick-up rate, and muscle contraction (Riveles et al., 2003, 2004, 2005). Smoke solution was first passed through solid phase extraction cartridges which were then eluted with methanol. The eluates were collected and tested in the beat frequency, oocyte pick-up, and muscle contraction assays. Three cartridges retained most of the inhibitory activity, and their eluates were analyzed by gas chromatography-mass spectrometry to identify the chemicals in each eluate. This produced a list of about 40 chemicals, most being pyridines, pyrazines, and phenols. Authentic standards of each chemical were purchased and tested individually in dose response experiments in each oviductal bioassay. From these data, a hierarchy of potency was created for each chemical in the beat frequency, pick-up rate, and muscle

contraction assays. Many of the chemicals were highly inhibitory in each assay in the nano- and picomolar range, and some inhibitory chemicals were not previously recognized as toxicants (e.g., 3-ethylpyridine and pyrazine). Some of the identified toxicants are added to tobacco to enhance its flavor and appear on the FEMA GRAS list (Flavor and Extract Manufacturers Association, generally regarded as safe) and on the FDA EAFUS list (everything added to food in the United States). Nicotine, an abundant highly bioactive molecule in smoke, did not produce significant effects in these assays unless used at high doses which were likely cytotoxic (Riveles et al., 2003). The observation that diverse assays (ciliary beating, oocyte pick-up rate, and muscle contraction) were inhibited by these toxicants suggests that they target a process fundamental to all cells or that they have multiple targets in cells. These data demonstrate the usefulness of the *in vitro* infundibular bioassays in studying the effects of smoke on oviductal functioning and in screening complex mixtures to identify specific toxicants.

#### *Uterus*

The uterus is a contractile organ and its endometrial lining undergoes cyclic changes important for implantation. Maintenance of an intact uterine lining and quiescent musculature throughout pregnancy is necessary to avoid spontaneous abortion. Placenta abruptio and premature deliveries are more common in smokers than nonsmokers, suggesting that the uterus is targeted by smoke inhalation (Andres and Day, 2000). Several *in vitro* assays have been used with the uterus in conjunction with tobacco smoke. These involve measuring muscle contraction in isolated strips of uterus, culturing uterine microvascular cells *in vitro*, or measuring contraction of isolated uterine arteries.

**In vitro contraction of uterine strips.** Women who smoke are at risk for preterm delivery (Meis

et al., 1995; Simpson, 1957) which could be caused by increased uterine contractions. It is possible to measure contraction *in vitro* using uterine strips. In a recent study on rat and human myometrium, force and frequency of contraction of uterine muscle was measured after 24 hr of exposure to cigarette smoke solution (Nakamoto et al., 2006). Oxytocin was used to induce contractions after smoke exposure. Both rat and human myometrium showed increased contractile force (but not frequency) in response to oxytocin, and real-time PCR showed that the expression levels of the oxytocin receptor were significantly higher in smoke treated uteri of both species. These interesting *in vitro* data suggest that chemicals in smoke make the uterus more responsive to oxytocin by increasing the number of oxytocin receptors, which could contribute to preterm deliveries often seen in women who smoke.

Several groups have used uterine strips to assess the effects of nicotine on uterine muscle contraction. Treatment of uteri from non-pregnant rabbits with nicotine leads to an increase in amplitude of electrical field stimulation evoked contraction (Nas et al., 2007). However, another group working with isolated mouse uterine horns found that nicotine inhibited electrically induced contractions, perhaps through action on the presynaptic nicotine receptors (Medina et al., 1992).

**Uterine microvasculature assays.** In addition to uterine muscle, the vasculature of the uterus has been examined *in vitro* in tobacco-related studies. Human uterine microvascular endothelial cells can be purchased and grown *in vitro* in the presence of smoke conditioned medium (Soghomonians et al., 2004). PECAM-1 is an important endothelial cell protein that functions in cell adhesion and may be important in endothelial cell migration. Cigarette smoke conditioned medium caused a reduction in the PECAM-1 band around endothelial cells and redistribution of all PECAM-1 to the cell surface.



Treatment also inhibited shear stress-induced migration of cells. This latter effect could be important if it occurs in vivo where failure of endothelial cells to migrate may affect uterine physiology and implantation during pregnancy.

#### **Uterine artery contractility.**

Uterine arteries isolated from near term pregnant sheep have been cut into rings, cultured in vitro, and used to measure contraction after chronic and acute exposure to nicotine (Xiao et al., 2007). While acute exposure did not affect contraction, chronic exposure to nicotine did cause a dose dependent enhancement of constriction of the uterine artery, which could impede blood flow to the uterus during pregnancy.

#### *Placenta*

The placenta is a transitory part of the reproductive system, present only during pregnancy. Numerous studies have shown that placentas in pregnant women are targets of cigarette smoke (van der Veen and Fox, 1982; Burton et al., 1989; Pfarrer et al., 1999; Salafia and Shiverick, 1999; Shiverick and Salafia, 1999; Zdravkovic et al., 2005; Jauniaux and Burton, 2007). Because placentas are readily available from hospitals, many in vitro studies have been done using human placental tissue. In fact, this is one model in which human tissue is used often for in vitro work on tobacco.

For proper and complete functioning, the human placenta relies on formation of floating villi which enable exchange of nutrients and gases between the maternal and fetal blood and anchoring villi which secure it to the uterine wall. Cytotrophoblasts from the implanting conceptus are essential for villi formation because their fusion leads to formation of the syncytium that covers floating villi. They continue to divide in anchoring villi to form cell columns that invade the uterus thereby securing attachment of the placenta to the mother. The placenta plays a vital role in prenatal development by

transporting nutrients and wastes between the maternal and fetal circulation and by providing hormones needed for normal development.

**Placental explants.** Human placental explants can be grown and studied experimentally in vitro (Genbacev et al., 1992). In one such study, it was shown that anchoring villi from smokers had difficulty undergoing cell column differentiation in vitro in comparison to control villi from nonsmokers (Genbacev et al., 1995). Moreover, nicotine alone was able to inhibit differentiation and thereby retard cytotrophoblast invasion in an in vitro assay. These authors further showed that nicotine inhibited synthesis and activation of type IV collagenase, which is necessary for cytotrophoblast invasion.

Some placental studies have involved an interesting correlation of in vivo and in vitro experimentation (Genbacev et al., 2000). For example, chorionic villi from women who smoked more than 20 cigarettes per day have morphologically defective floating and anchoring villi which were characterized by a decreased number of Ki67 positive cells, indicative of decreased mitotic activity in the cytotrophoblasts of the villi (Genbacev et al., 1995; Genbacev et al., 2000). Nicotine was subsequently identified as a chemical in smoke contributing to inhibition of mitosis by culturing anchoring villi from nonsmokers in vitro in media containing nicotine. This experiment resulted in a decreased expression of cell cycle markers and decreased incorporation of BrdU, implicating nicotine in retardation of placenta growth in smokers (Genbacev et al., 2000). These authors further showed that expression of two markers characteristic of normal cytotrophoblast differentiation (fibronectin and its  $\alpha 5 \beta 1$  integrin receptor) were also reduced during in vitro exposure to nicotine. These data demonstrate that nicotine is a key molecule in tobacco that inhibits the growth

and differentiation of cytotrophoblasts in the human placenta.

In a subsequent study by the same group, L-selectin, which functions initially in attachment of the embryo to the uterus and later in attaching anchoring villi to the endometrium, was examined in smokers and nonsmokers (Zdravkovic et al., 2006). Immunohistochemical results showed less L-selectin in villi of smokers. Nicotine was shown using video microscopy of in vitro cultures to inhibit outgrowth of cytotrophoblasts from cell columns in anchoring villi. The above studies together demonstrate that nicotine impairs growth, differentiation, and attachment of cytotrophoblasts and thereby produces important harmful effects on human placentas.

#### **In vitro placental perfusion.**

Term placentas have been used in vitro with a perfusion system that allows pressure within the placental vasculature to be monitored during experimental treatment (Bainbridge et al., 2002). When nicotine was perfused into this system at doses as high as 240 ng/ml, no change in vascular pressure was observed (Bainbridge and Smith, 2006). This negative result is interesting since it has been thought for many years that nicotine reduces fetal birth weight by constricting the placental vasculature, but this now seems not to be the case.

**Placental microsomes.** Microsomes can be prepared from human placentas and used to evaluate placental metabolism in vitro. In one such study using term placental microsomes, nicotine, cotinine, and anabasine inhibited conversion of testosterone to estrogen (Barbieri et al., 1986a). In some studies, placental microsomes from smokers and nonsmokers have been compared. Interindividual variation in response to benzo[a]pyrene or in the ability to detoxify have been found to be high (Sanyal and Li, 2007).

#### *Umbilical Cord*

The umbilical cord contains two arteries and one vein that transfer

nutrients and wastes between the maternal and fetal circulation. The endothelial cells lining the vein (human umbilical vein endothelial cells, HUVEC) are readily obtained either by direct isolation from fresh cords or by purchase from commercial vendors. They can be grown *in vitro* in monolayer cultures and have been attractive models for studies involving tobacco smoke. One drawback to this system is that cells may vary from batch to batch. Various assays can be performed with HUVEC that provide important information on angiogenic processes. These include assays for measuring proliferation, apoptosis, tube formation, and migration.

**Human umbilical vein endothelial cell cultures.** Human umbilical vein cells (HUVEC) have been used in numerous studies with smoke extracts or solutions, and much of this work has been reviewed recently (Ambrose and Barua, 2004). In general, exposure of HUVEC to cigarette smoke has produced detrimental effects, including reduced migration (Snajdar et al., 2001) and induction of apoptosis (Wang et al., 2003; Yang and Liu, 2004).

Individual chemicals in smoke have also been examined using HUVEC. Six chemicals that were highly inhibitory in the oviductal bioassays were tested using HUVEC and adult microvascular endothelial cells (HMVEC) (Yu et al., 2006). Survival of both endothelial cell types was strongly impaired by ethylpyridines and pyrazine and moderately impaired by *p*-cresol, with HUVEC being more sensitive than adult HMVEC. Nicotine, in contrast, stimulates growth of endothelial cells, including HUVEC *in vitro*, (Villablanca, 1998; Heesch et al., 2001; Yu et al., 2006) and induces tube formation in HUVEC (Heesch et al., 2001). These studies show that HUVEC can respond quite differently to different chemicals in smoke. The ability of nicotine to stimulate endothelial cell growth has implications in tumor formation *in vivo* (Heesch et al., 2001).

### Embryos

Chemicals in smoke transfer readily across the placental membrane and may reach high concentrations in the amniotic fluid (Jordanov, 1990). The embryo and fetus are vulnerable to environmental toxicants and receive significant exposure to chemicals in smoke during pregnancies in active and passive smokers.

**In vitro culture of postimplantation embryos.** *In vitro* culture of rat embryos has been used to evaluate the effect of nicotine on embryonic development and for teratological screening (Joschko et al., 1991). Rat embryos were removed from pregnant uteri on day 9.5 and cultured in the presence or absence of various doses of nicotine for 48 hr. Evaluations of growth and morphology were done using light and electron microscopy. Data showed that nicotine retarded growth and produced developmental abnormalities in the forebrain and branchial arches. In a subsequent study using embryo culture of mouse embryos, nicotine was shown to induce apoptosis in both the brain and spinal cord (Zhao and Reece, 2005).

**Growth and differentiation of embryonic stem cells.** Embryonic stem cells provide a valuable *in vitro* system for studying the effects of smoke on the early stages of development and differentiation. Embryonic stem cells give rise to all cells in the embryo and affects by smoke could have dire consequences. Embryonic stem cells can be used to examine the effects of smoke on survival and growth of stem cells or on their differentiation into various lineages. Both mouse and human embryonic stem cells are available for use in tobacco-related studies (Martin, 1981; Thomson et al., 1998). In preliminary work with assays using mouse embryonic stem cells, whole mainstream and sidestream smoke inhibited growth of mESC, with sidestream smoke being the more potent (Lin et al., 2007). Moreover, smoke from one

harm reduction brand was more inhibitory than smoke from a traditional cigarette. Further development of embryonic stem cells for testing of smoke will add an important *in vitro* assay for studying smoke's effect on early development.

### CONCLUDING REMARKS

An array of procedures is available for studying reproductive and developing organs *in vitro*. These assays have provided basic knowledge about the functioning of the reproductive organs and have enabled toxicological studies on each organ. *In vitro* studies using cigarette smoke or its chemicals have helped identify organ, cellular, and molecular targets of smoke in the reproductive system, define the type of harm done by tobacco products, screen and identify toxicants in smoke, and in some cases identify the mechanism of action of smoke components. Perhaps, one of the most remarkable features of the *in vitro* work is how many different reproductive processes have been shown to be affected by cigarette smoke. In the ovary alone, oocyte survival, follicular growth, steroid synthesis, oocyte maturation, zona pellucida formation, and cumulus expansion are all inhibited by smoke or specific components in smoke. In the future, improved and expanded *in vitro* assays can be further applied to the study of tobacco smoke's effect on reproduction. The development of new complementary assays to study additional properties of the reproductive system and to improve the existing assays will be helpful in furthering our understanding of smoke's interaction with the reproductive organs.

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