Vascular and Morphogenetic Abnormalities Associated with Exposure of Cigarette Smoke Condensate during Chicken and Murine Embryogenesis[1]

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Objective Embryonic movements (EM) and angiogenesis pathways are evolutionarily conserved mechanisms which are essential for proper embryonic development. Deviations in these processes by exposure to cigarette smoke condensate (CSC) may cause vascular and morphogenetic disorders. **Methods** Using chicken and mouse embryos, we have demonstrated the *in vivo* effects of CSC on EM, vascular development, and organogenesis. **Results** Examination of the CSC exposed chicken embryos revealed a significant reduction in EM, stunted growth, deviated pattern of blood vessels, hemorrhages, and localized necrosis. Likewise, mouse embryos that were exposed to CSC at E8.5 and E9.5 died between E11.5 and E12.5, respectively. These mouse embryos showed defects in morphogenesis and remodeling of the embryonic vasculature, while littermate controls showed normal development. **Conclusion** Cigarette smoking during pregnancy is fatal for growing embryos. CSC may induce the remodeling of embryonic vasculature, leading to various pathologies.

Key words: Angiogenesis; Embryonic movements; Cigarette smoke condensate; Vascular remodeling

INTRODUCTION

Angiogenesis, the development of new capillaries from pre-existing vessels, is a vital physiological process which is induced by inflammation, wound healing, and immune reactions[1]. In the normal ovulating female, ovulation, formation of the corpus luteum, cyclic regeneration of the endometrium, and embryonic development all involve angiogenesis[2]. Pathological angiogenesis, often referred to as neovascularization, is associated with several disease conditions, including retinopathies, arthritis, psoriasis, and cancer[3]. Recent investigations have broadened the study of angiogenesis into a distinct field of scientific inquiry which has yielded insights into the regulation of vascular growth. It is now well established that the process of angiogenesis is fundamental for growth and development of an embryo[4]. Any obstruction to this physiological process may produce a number of pathological conditions which are fatal for a growing embryo.

Cigarette smoke is a complex mixture of over 4 500 toxic substances[5-6]. Exposure to cigarette smoke is a major preventable risk factor for several diseases, including psoriasis[5], cataracts[6], skin wrinkling[7], deafness[8], oral cancer[9], tooth decay[10], emphysema[11], heart diseases[12], stomach ulcer[13], osteoporosis[14], skin discoloration[15], and cervical cancer[16]. In addition, numerous epidemiological studies have shown that women who smoke cigarettes can experience reproductive problems, including ectopic pregnancy[17]. The public is generally aware of the harm done to the lungs and circulatory system by exposure to cigarette smoke[11]; however, the effects of smoke on a growing fetus or embryo are not well understood. There are three primary types of exposure to cigarette smoke, which include mainstream cigarette smoke (inhaled by the smoker), side stream cigarette smoke (inhaled by non-smokers in places where smoking is allowed), and cigarette smoke condensate (CSC). CSC represents sticky particles which are comprised of thousands of chemicals created by burning tobacco[18].

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In our previous studies, we reported on the different detrimental effects of mainstream cigarette smoke solutions (MSCSS) and second-hand cigarette smoke solutions (SSCSS) on EM[19-20] and angiogenesis[21-22]. In a continuation of our previous studies, the current experiment attempts to evaluate the various toxic effects on angiogenesis and organogenesis of cigarette smoke condensate (CSC) found in commercially available cigarettes.

Testing the undesirable effects on fetal movements and embryonic development has turned into a vital part of toxicological studies[23]. These studies involve applying the drug to be tested on at least two species of animals. Outcomes are appraised according to the incidences of malformed fetuses. This experiment involves a method for testing the embryotoxicity of CSC using chicken embryos and pregnant mice. The present method requires observing the response of the vascular and morphogenetic system in the chicken and mouse embryos 24 h after the application of the compound being tested.

MATERIALS AND METHODS

Preparation of Chicken Embryos

Fresh fertilized eggs of similar weights were obtained from a local hatchery. All of the eggs were sprayed with 70% ethanol and air-dried to reduce contamination from the egg surface. The eggs were then incubated at 37 ℃ for four days and windowed aseptically as described by Ejaz[24], with slight modifications. A small window (approximately 2cm in diameter) was made by removing the shell and inner shell membrane from the air-space site. On the same day, 2.5 mL of albumin was aspirated with a sterile 21 G cannula to allow the embryos to develop in a way accessible to holistic quantification. The windows were then sealed with sterile parafilm tape (American National Can, Chicago, IL, USA), and the eggs were returned to the incubator at 37 ℃ (humidity 55%-60%). They were then incubated with the window upright until day six of incubation, when the test materials were placed on the developing embryos.

Preparation and Administration of the Tobacco Smoke Condensate Solution

A market survey was carried out to determine the most commonly smoked commercial cigarettes. They were then used to prepare CSC. CSC was prepared immediately before use, following the method described by Kim and Luppi[25-26]. CSCs were collected onto a Cambridge filter pad. After sterilization by EO gas, these pads were placed on the embryos on day six of incubation.

Video Recordings and Kinematic Analyses

On day six of incubation, all of the eggs were transferred to an incubator, fitted with a camera, and maintained at a temperature of 37 ℃ and a relative humidity of 85%. All of the CSC disks were also placed in the same environment in order to prevent temperature shock to the embryos. Before the application of the disks, all of the eggs were placed in the incubator for 30 min to acclimatize them to the environment. The EM of each egg were recorded for 60 min. At minute 15, the CSC disks were applied to each embryo.

The video recordings were continuous in order to capture all movements and pauses in movements over the entire experiment for each embryo. The videos were recorded at 30 frames/sec, using a camera shutter speed of 1/2000 s. The videos were saved on a computer hard disk. For greater precision, control data for each group was first examined to evaluate the normal EM. These data were then used to determine the activity which was altered by the application of CSC.

In order to determine the onset and termination of activity, two reviewers did a frame-by-frame evaluation of the saved recordings (Fig. 1). The beginning and end of each motility sequence, as well as the subsequent pause, were determined during video playback. By adapting methods for direct observation established by Hamburger and colleagues[27], a motion sequence was defined as the continuous movement of the right wing and/or leg. A pause in motion was defined as the absence of detectable excursions. The left limbs were ignored because they were usually out of view. A span of 3 min was selected for the evaluation of EM.

Administration of Cigarette Smoke Condensate to Pregnant Mice

The administration of CSC to pregnant mice was carried out exactly as described elsewhere[28]. Pregnant mice were treated with CSC on days 8.5 and 9.5 of gestation. The dose of CSC administered to the mice was 15 mg/kg of body weight. Because mice are obligate nose breathers, insufflations were used to administer CSC. Control mice were treated in the same fashion using DMSO in place of CSC. All of the animals were anesthetized 24 h post dosing. The embryos were collected for whole mount staining with PECAM.

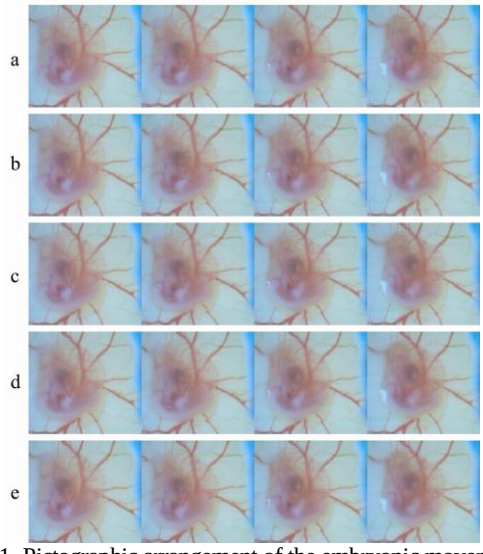


FIG. 1. Pictographic arrangement of the embryonic movements of chicken embryos which were exposed to cigarette smoke condensate. The end of the experiment, the initiation and end of each motility sequence, and the subsequent pause was determined during video playback. A span of 3 min was selected for the evaluation of embryonic motion. Note the differences in the position of the embryos in panels a, b, c, d, and e.

Whole Mount Staining of Embryos with PECAM

The C57BL/6J pregnant mice were sacrificed on day 9.5 and 10.5 of development. The embryos were dissected and washed three times in PBS, then fixed in cold 2% paraformaldehyde/20 mmol/L sodium phosphate at pH 7.4 for 30 min. They were then rinsed three more times in PBS. The embryos were blocked in PBS containing 3% skimmed milk (Bio-Rad Laboratories, Hercules, CA) and 0.05% Triton X-100 for 1 h. The whole-mount embryos were incubated with anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) antibodies (clone MEC13.3, PharMingen), and then with alkaline phosphatase-conjugated secondary antibodies (Promega) as previously described[29]. Embryos were mounted in 70% glycerol/PBS.

Stereoscopic Evaluation of Growth Defects in CSC Exposed Embryos

All of the embryos which were exposed to CSC were macro- and stereoscopically screened 24 h after exposure for any vascular and/or morphogenetic growth defects. Disorders in the development of the facial region, extremities, cranial vault and caudal end of the trunk, malformations of the heart and abdominal wall were evaluated.

Statistics

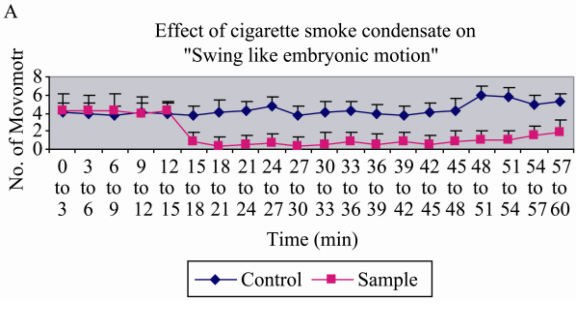
An ANOVA was performed in order to evaluate the differences in the number of EM between the control and treated samples during each 3 min interval. Statistical significance was set at $P<0.05$. A student's *t*-test was also performed to compare the average sums of the different EM between the treated and control chick embryos at each 3 min interval[29].

RESULTS

Cigarette Smoke Condensate Reduced Embryonic Movements

Four types of EM were recorded during the course of the experiment. They included a swing-like motion, motion of the head, motion of the tail, and motion of the entire body. A decrease in all four types of EM was observed in the control groups shortly after the application of normal saline at 15 min. The recovery phase began at 18 min. The swing-like motion was significantly decreased ($P<0.001$) after exposure to CSC. A small recovery from the baseline EM was recorded. However, normal EM did not recover until the end of the experiment (Fig. 2a). The application of CSC to the developing embryo caused a significant decrease ($P<0.001$) in the EM of the head until the end of the experiment. The complete cessation of head movement was observed at 15-18 min. The recovery phase started at 30-33 min, and normal movements did not recover until 60 min of recording (Fig. 2b).

A significant decrease ($P<0.001$) in the EM of the tail was recorded after application of the CSC. The movement became negligible at 33-36 min. The recovery phase of the tail motion began at 45-48 min, and gradually increased. However, complete recovery was not observed until 60 min of recording (Fig. 2c). Likewise, the application of CSC to the embryos also resulted in the rapid decline and a complete pause ($P<0.001$) in the motion of the entire body. The recovery phase started after 39-42 min, however no significant recovery was observed until 60 min (Fig. 2d).



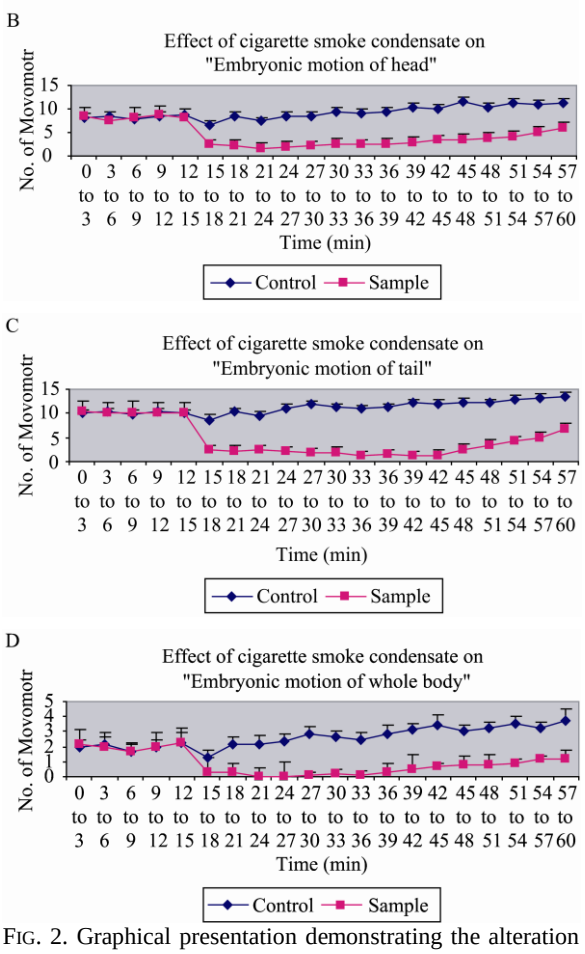


FIG. 2. Graphical presentation demonstrating the alteration in the embryonic movements following exposure to cigarette smoke condensate. A significant decrease (P<0.001) in all four embryonic movements was recorded after the application of CSC to the developing embryos. CSC maximally affected the swing-like motion and motion of the tail. The recovery phase was always between 45-48 min.

The application of CSC to the developing embryos caused a significant decrease (P<0.001) in all four categories of EM. CSC affected the swing-like motion and the motion of the tail more than the other types of motion. For these motions, the recovery phase was always between 45-48 min.

Cigarette Smoke Condensate Caused Delayed Growth and Morphogenetic Deformities in Chicken Embryos

The chicken embryos were collected 24 h after CSC exposure and microscopically analyzed for morphogenetic and vascular deformities. All of the treated embryos had stunted growth (Fig. 3a, b) compared to the control embryos (Fig. 3c). On embryonic day 7 (E7), cranial hemorrhage was evident in nearly all of the CSC treated embryos. In addition, embryos also had widespread hemorrhage in the orbital areas (Fig. 3d, e), mandible and neck areas, spinal cord and pericardial areas, with significant hemorrhages in the abdominal area (Fig. 3d, e). Beak deformities were observed in most of the CSC treated embryos (Fig. 3d). These results are strongly reminiscent of the morphogenetic and vascular deformities seen in the CSC exposed embryos.

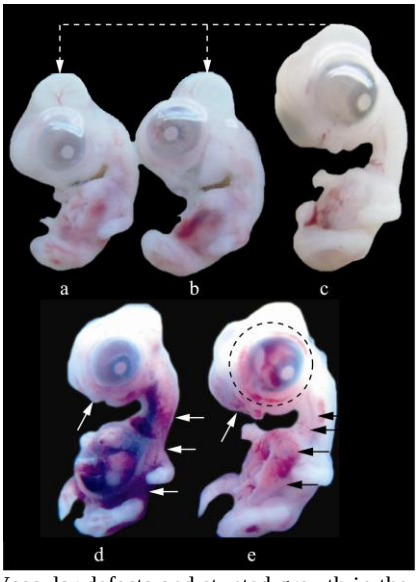


FIG. 3. Vascular defects and stunted growth in the cigarette smoke condensate exposed chicken embryos. (a,b,d,e) Photomicrographs of E7 embryos showing retarded growth and hemorrhaging in the head, periorbital, mandible, spinal cord, and abdominal regions. Defective beak development was also a prominent finding among the CSC treated embryos.

Mouse Embryos Exposed to Cigarette Smoke Condensate Exhibit Defects in Vascular Remodeling and Organogenesis

On embryonic day 9.5, the embryos were clearly showing growth retardation (Fig. 4b) after exposure to CSC. These embryos were clearly distinguished from their wild-type littermates (Fig. 4a). Identical morphogenetic defects were true while examining embryos at E 10.5 (Fig. 5). To determine when the CSC exposed embryos were dying, the embryos were isolated after exposure. Exposure of the mouse embryos to CSC at E8.5 and 9.5 resulted in embryonic lethality between E11.5 and E12.5, respectively. There were defects in morphogenesis and remodeling of the embryonic vasculature. Littermate controls showed normal development. On embryonic day 10.5, the embryos could be identified easily due to hemorrhaging, particularly in the cranial region. When isolated at E12.5, the embryos were either completely resorbed, or were severely necrotic.

We visualized the vascular network of the CSC treated embryos and littermate controls in whole mount preparations by staining them with a monoclonal antibody PECAM-1. This is a specific marker for vascular endothelial cells[30]. Vascular growth was prominently impaired in the cranial region of E9.5 CSC exposed embryos. Close observation of the capillary head plexus (Fig. 4d) and tail somites (Fig. 4f) development revealed reduced and delayed vascular development in the CSC treated embryos. In contrast, the control littermates displayed a well-developed capillary head plexus (Fig. 4c), as well as tail somite development (Fig. 4e). In addition, fully vascularized heart and limb-buds (Fig. 4e) were prominent findings in the wild type embryos. This served to highlight the significant retardation in the vascular remodeling of the CSC treated embryos.

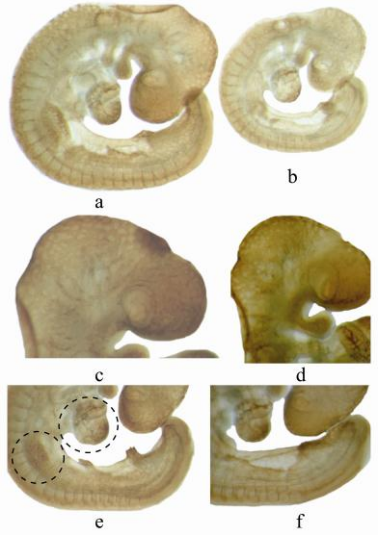


FIG. 4. Wholemount staining of E9.5 embryos from cigarette smoke condensate exposed pregnant mice. Impaired vasculature growth was observed in the capillary head plexus (d) and tail somite regions (f). In contrast, control littermates displayed excellent growth of the capillary head plexus (c) and tail somites (e). Note the reduced embryo size (b) and delayed growth of the heart (f) with missing limb buds in the CSC treated embryos.

The vascular network overlying the forebrain of the CSC exposed E10.5 embryos was not as intricate (Fig. 5b) as that of the wild-type littermates (Fig. 5a). The CSC treated embryos (Fig. 5d) had an abnormal appearance of the venae capitis, with a reduced spread and diameter. Maturation of the dorsal aorta was delayed in the CSC treated embryos (Fig. 5b), while control littermates showed a ramified vascular network with numerous capillary branches emerging from segmental vessels (Fig. 5a). Atrioventricular compartments and limb buds were fully vascularized in the wild type embryos (Fig. 5a), while less vascularized atrioventricular compartments and missing limb buds were prominent findings in the CSC treated group (Fig. 5b).

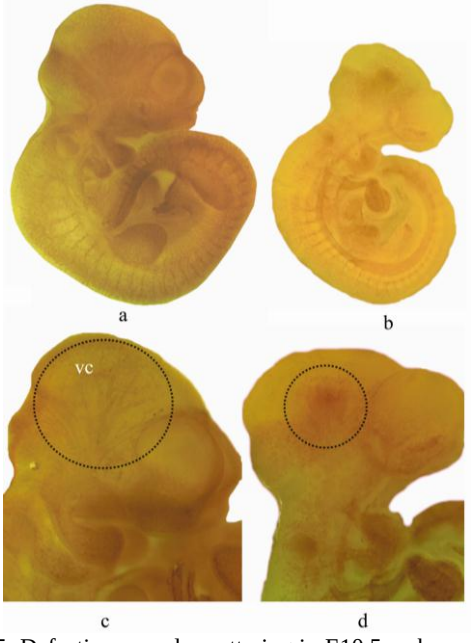


FIG. 5. Defective vascular pattering in E10.5 embryos from cigarette smoke condensate exposed pregnant mice. (a) Control littermates showing a ramified vascular network with numerous capillary branches emerging from segmental vessels, atrioventricular compartments, and limb buds. Maturation of the dorsal aorta is delayed in the CSC treated embryos (b). (d) Atypical manifestation of the venae capitis (vc) of the head with abridged spread and diameter in the CSC treated embryos.

DISCUSSION

The present study has shown that exposure to CSC during embryonic development is detrimental to EM and angiogenesis. EM and angiogenesis serve to control the development and maturation of the developing organs. In this study, chicken embryos were used as an alternative *in vivo* approach. This allows for the continuous visualization of the implant area, while providing rapid, simple, and low-cost screening of tissue reactions to different toxic materials. The mammalian placenta is an evolutionary homolog of the chick chorioallantoic membrane, and CAM-yolk interactions are analogous to that of the placenta and maternal blood[31-32].

In a study of embryonic chick movements, Hamburger and Oppenheim[33], used direct observational methods to identify three forms of behavior *in ovo*. They are referred to as type I, II, and III movements. A type I EM is defined as a random, jerky, small amplitude movement. A type II EM is

defined as a sudden, rapid wriggle and startle of the entire body. Observations indicated that instances of type II EM were followed by a type I EM. A type III EM is defined as a pre-hatching and hatching EM (tucking and piping of the egg shell), and is viewed as the first coordinated, goal-oriented behavior produced by the embryo. Their findings indicate that type I movements initiate with the onset of EM on days 3.5 of incubation, and continue through the end of incubation. Type II movements began on day 11 of incubation and type III movements began on day 17 of incubation. The above definitions suggest that each movement is a unique behavior with a distinct developmental time course. Thus, any changes in these movements have the potential to hinder the normal process of embryogenesis[34].

Our results clearly demonstrate that application of CSC to developing embryos significantly depressed (P<0.001) all four EM. Maximal effects were observed on the swing-like motion and motion of the tail (Fig. 2). These are vital for organogenesis. In addition, a microscopic examination of the same CSC exposed embryos revealed delayed growth with abnormal vascular development and hemorrhaging around the entire body of the embryo (Fig. 3). Identical results were obtained in the embryos of pregnant mice after exposure to CSC (Figs. 4 and 5). Exposure of mouse embryos to CSC at E8.5 and 9.5 resulted in embryonic lethality between E11.5 and E12.5, respectively. The causes of these deaths included defects in morphogenesis, and remodeling of the embryonic vasculature. A wholemount PECAM explained the defective vascular patterning in the cranial and somite regions of the E9.5 and E10.5 embryos which were exposed to CSC (Figs. 4 and 5). Additionally, abnormal growth of the venae capitis (Fig. 5d) and delayed maturation of the dorsal aorta were commonly observed in the E10.5 embryos which had been exposed to CSC (Fig. 5b).

Smoking during pregnancy is known to interfere with the normal process of angiogenesis. This results in an increased incidence of ectopic pregnancy, spontaneous abortion, preterm delivery, sudden infant death syndrome, and delayed wound healing[20]. The use of tobacco metabolites by pregnant women is associated with fetal tobacco syndrome[35]. CNS involvement, which is characterized by developmental delay, is seen in at least fifty percent of cases of fetal tobacco syndrome in children of mothers who smoke more than 20 cigarettes per day[36]. In addition, children of cigarette smokers have shown deficits in growth, intellectual and emotional development, and behavior[37-38].

Despite the known health hazards of cigarette smoking, there is a surprising lack of experimental data regarding the developmental toxicity of the more than 4 500 smoke constituents[39]. The findings of our experiment demonstrate, for the first time, that exposure to CSC is very risky during pregnancy. It has potential effects on several underlying developmental events. Interruption of these events can result in vascular and morphogenetic abnormalities in growing embryos.

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