Disentangling prenatal and inherited influences in humans with an experimental design

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Exposure to adversity in utero at a sensitive period of development can bring about physiological, structural, and metabolic changes in the fetus that affect later development and behavior. However, the link between prenatal environment and offspring outcomes could also arise and confound because of the relation between maternal and offspring genomes. As human studies cannot randomly assign offspring to prenatal conditions, it is difficult to test whether in utero events have true causal effects on offspring outcomes. We used an unusual approach to overcome this difficulty whereby pregnant mothers are either biologically unrelated or related to their child as a result of in vitro fertilization (IVF). In this sample, prenatal smoking reduces offspring birth weight in both unrelated and related offspring, consistent with effects arising through prenatal mechanisms independent of the relation between the maternal and offspring genomes. In contrast, the association between prenatal smoking and offspring antisocial behavior depended on inherited factors because association was only present in related mothers and offspring. The results demonstrate that this unusual prenatal cross-fostering design is feasible and informative for disentangling inherited and prenatal effects on human health and behavior. Disentangling these different effects is invaluable for pinpointing markers of prenatal adversity that have a causal effect on offspring outcomes. The origins of behavior and many common complex disorders may begin in early life, therefore this experimental design could pave the way for identifying prenatal factors that affect behavior in future generations.

Events occurring in prenatal life may have long lasting effects on behavior and health (1, 2). However, many important prenatal risk factors that impact on offspring development in utero are influenced by maternal characteristics (3–7). Consider the example of smoking in pregnancy, which is associated with a number of adverse outcomes in offspring including reduced birth weight and increased antisocial behavior. Plausible physiological mechanisms have been suggested to explain these associations (8–11). However, smoking in pregnancy is influenced by maternal characteristics as well as by maternal genotype (6, 8, 12). Therefore, in the absence of random assignment of offspring to prenatal environments, associations between prenatal smoking and offspring outcomes could arise through maternally provided inherited factors as well as true “prenatal effects”.

We focus on 2 key adverse offspring outcomes associated with maternal smoking in pregnancy: (i) reduced birth weight and (ii) increased levels of childhood antisocial behavior (8, 9). Both outcomes are common and areas of significant societal concern: Low-birth-weight infants have high rates of chronic conditions and special health needs with associated health care costs (13). Individuals who are antisocial as children show a range of social and achievement impairments and increased rates of crime in adult life (14). Studies of animals (10, 11) and epidemiological cohorts (15) suggest that tobacco smoking during pregnancy likely has a causal risk effect on reduced birth weight. There is uncertainty about why there are links between maternal smoking during pregnancy and offspring antisocial behavior, both of which are heritable phenotypes (12, 16). Some studies find that associations persist when confounding factors are included (8, 9), although others suggest that the association may be driven by maternal characteristics (6, 17). Disambiguating the reasons why prenatal smoking is associated with offspring antisocial behavior has important public health implications (6, 7, 9). However, the difficulty of disentangling prenatal effects from inherited influences is not easily solved except through experimental methods that to date have only been possible in animal studies.

Prenatal cross-fostering in animals permits a test of the relative contributions of maternally provided prenatal and inherited factors (18, 19). Until recently, it was unimaginable that it might be possible to assess human offspring whose prenatal environment is provided by an unrelated mother. This research is now theoretically feasible given the increasing use of in vitro fertilization (IVF) as a means of conception (20). Children conceived via these methods may be related to both parents (homologous IVF), the mother only (IVF with sperm donation), the father only (IVF with egg donation), or to neither parent (IVF with embryo donation). Thus, with egg and embryo donation, the woman who experiences the pregnancy is not biologically related to the fetus. Therefore, if an association between prenatal adversity and offspring outcome is seen in this group, it must arise through prenatal effects independent of influences inherited from the mother.

The records of 779 children born through IVF, whose prenatal environment was provided by either a related mother or an unrelated mother, were examined. We assessed the links between exposure to tobacco smoking in pregnancy and offspring (i) birth weight and (ii) antisocial behavior in related and unrelated pregnancies. The key analytical step was to test for association between prenatal smoking and child outcome in the group where the woman experiencing the pregnancy was unrelated to the child (n = 208). If there is significant association in the unrelated group, this association cannot be attributable to inherited factors from the mother. Thus, association in the unrelated group must arise because of prenatal effects independent of the relation between maternal and offspring genome. In contrast, when association is only seen in the related group, this indicates that heritable factors account for the association. In this design, such genetic dependence will include inherited DNA.
sequence variation, and potentially, also epigenetic variation. Only association that is independent of inherited influences is consistent with a causal role for prenatal adversity (after implantation) in the development of offspring outcome/behavior. Table 1 shows descriptive characteristics of the sample. Information on prenatal smoking and offspring outcomes was obtained from maternal questionnaire and antenatal records. In terms of child birth weight and antisocial behavior, the present sample did not differ substantially from normative comparison data (see Methods) (21, 22). Forty-six women (6%) smoked during pregnancy. There was no difference in the rate of smoking in the related and unrelated groups ($\chi^2 = 1.16, df = 1, P = 0.28$).

**Results**

**Offspring Birth Weight.** Mothers who smoked in pregnancy had lower birth-weight offspring ($t = -3.08, df = 767, P = 0.002$, smoker-offspring mean = 2,771 grams, nonsmoker-offspring mean = 3,091 grams). This was the case in both the related ($F = 15.26(1,473), P = 0.001$, partial $\eta^2 = 0.031$) and unrelated pregnancies ($F = 6.64(1,168), P = 0.01$, partial $\eta^2 = 0.038$). The pattern of results remained the same when only singleton births were included in the analysis (related group: $F = 14.28(1,371), P = 0.001$, partial $\eta^2 = 0.037$; unrelated group: $F = 6.12(1,131), P = 0.02$, partial $\eta^2 = 0.045$). As expected, the interaction between group (related/unrelated) and prenatal smoking was nonsignificant ($F = 0.857(1,647), P = 0.355$, partial $\eta^2 = 0.004$). Fig. 1 shows standardized means for birth weight (adjusted for covariates and gestational age) in the related and unrelated offspring of smokers and nonsmokers. Fig. 1 illustrates that smoking in pregnancy was significantly associated with a reduction in birth weight in both related and unrelated offspring. This pattern of results is therefore consistent with prenatal mechanisms explaining the effect of prenatal smoking on offspring birth weight.

**Offspring Antisocial Behavior.** In the sample as a whole, mothers who smoked had offspring with significantly higher antisocial behavior ($t = -2.39, df = 769, P = 0.02$, smoker-offspring mean = 2.40, nonsmoker-offspring mean = 1.84). However, examining the related and unrelated pregnancies separately showed that smoking during pregnancy was not associated with increased levels of offspring antisocial behavior in the unrelated group ($F = 0.157(1,184), P = 0.693$, partial $\eta^2 = 0.001$). Association was found only in the related group ($F = 7.93(1,145), P = 0.001$, partial $\eta^2 = 0.015$). The pattern of results is therefore different in the related and unrelated groups: Whereas offspring antisocial-behavior scores were higher in mothers who smoked in the related group (smoker mean = 2.54 ± 1.52, nonsmoker mean = 1.75 ± 1.48), this was not the case in the unrelated group (smoker mean = 1.77 ± 1.34, nonsmoker mean = 2.08 ± 1.60). These results therefore point to the importance of inherited factors in the association between prenatal smoking and offspring antisocial behavior and suggest that gene–environment correlation is important in explaining this association (7). These inherited factors are not indexed by maternal antisocial behavior because this variable was included as a covariate in analyses. As expected, the interaction between offspring relatedness and prenatal smoking was significant ($F = 4.106, P = 0.04$, partial $\eta^2 = 0.006$). Fig. 2 shows standardized means adjusted for covariates for prenatal smokers and nonsmokers in the related and unrelated pregnancies and illustrates that prenatal smoking did not significantly influence antisocial behavior in the unrelated offspring. Thus, prenatal mechanisms did not account for the link between maternal smoking in pregnancy and elevated antisocial behavior independent of factors inherited from the mother, although it is noted that the standard deviation is quite large because of the small number of unrelated smokers.

<table>
<thead>
<tr>
<th>Sample characteristics</th>
<th>Mother genetically related to fetus</th>
<th>Mother genetically unrelated to fetus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoked during pregnancy, yes/no</td>
<td>37/533</td>
<td>9/195</td>
</tr>
<tr>
<td>Child birth weight: mean ± SD, grams</td>
<td>3082.17 ± 674.86</td>
<td>3053.36 ± 735.94</td>
</tr>
<tr>
<td>Child gestational age: mean ± SD, weeks</td>
<td>38.42 ± 2.51</td>
<td>37.78 ± 2.86</td>
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<td>Family income mean, U.S. dollars</td>
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</tr>
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<td>Socio-occupational classification of main</td>
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<td>14</td>
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<tr>
<td>Mother earner, % manual</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child antisocial behavior score: mean ± SD</td>
<td>1.80 ± 1.50</td>
<td>2.05 ± 1.60</td>
</tr>
<tr>
<td>Child age: mean ± SD, years</td>
<td>6.81 ± 1.27</td>
<td>6.46 ± 1.29</td>
</tr>
<tr>
<td>Percentage singleton births, %</td>
<td>78</td>
<td>76</td>
</tr>
</tbody>
</table>

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Fig. 1. Reduction in offspring birth weight corrected for gestational age in related and unrelated offspring of prenatal smokers and nonsmokers. Standardized means, adjusted for covariates, are presented.

Fig. 2. Increased offspring antisocial behavior in related, but not unrelated, offspring of prenatal smokers and nonsmokers. Standardized means, adjusted for covariates, are presented.
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There were no gender differences in the patterns of association between prenatal smoking and child outcomes.

Discussion

Experimental methods are able to disentangle causal pathways (7). To date, prenatal cross-fostering studies have only been possible in experimental animal studies. Here, we use a similar approach, made possible by the increased use of IVF as a means of conception (20), to disentangle prenatal effects from inherited effects on offspring birth weight and antisocial behavior.

Effective public health strategies for reducing maternal smoking remain a key target because of the deleterious effects of smoking on fetal development (8–11) and a range of obstetric and perinatal complications, including premature birth and spontaneous abortion (23). Our results indicate that smoking in pregnancy has true prenatal effects on offspring birth weight, indicating that interventions aimed at reducing smoking in pregnancy may additionally change this outcome. Biological mechanisms proposed to explain the reduction in birth weight seen in infants exposed to prenatal smoking include nicotine toxicity and pharmacological effects of nicotine such as vasoconstriction and carbon monoxide exposure. Results of a recent experimental animal study suggest that exposure to carbon monoxide may be a major contributing factor to lower fetal weight in offspring exposed to prenatal smoking (24).

This new research design indicates that findings differ for offspring antisocial behavior where the association with prenatal smoking appears to be entirely explained by inherited pathways that are not attributable to maternal antisocial behavior. This pattern of results is therefore inconsistent with a causal risk effect, suggesting that interventions aimed at reducing prenatal smoking are unlikely to have effects on offspring antisocial behavior. It is noteworthy that this direct “experiment” appears to provide a powerful method of disambiguating developmental pathways in relation to current paradigms. For example, previous behavior genetic studies have not been able to provide clear data about the pathways underlying the association between prenatal smoking and offspring antisocial behavior despite having large sample sizes (6).

Although the children in this study were conceived by using IVF, numerous studies have shown that children conceived via IVF are similar in terms of psychological adjustment to children who are conceived naturally (25–27), although some studies report possible epigenetic alternations in this group (28). The present sample is also comparable to population-based data in terms of child and parent mental health (21, 29).

The best evidence for generalizable results will come from consistency of findings with studies involving naturally conceived offspring that show association between maternal smoking in pregnancy. Our findings for prenatal smoking and birth weight concur with results from experimental animal studies (10, 11, 24) and studies of naturally conceived children (15). The existing literature for prenatal smoking and offspring antisocial behavior is less clear than that for birth weight, highlighting the need for novel designs in this area. Nonetheless, some existing studies have suggested that the link between prenatal smoking and offspring antisocial behavior may not be causal (6, 17). Another consideration is that fetal genes could have effects on the prenatal milieu in unrelated pregnancies. However, fetal effects will not affect patterns of association between fetal outcome and maternal behavior in pregnancy, because they will be inherited in a random fashion from unrelated oocyte/embryo donors.

To conclude, the rapid uptake of IVF as a method of conception means that adoption studies which begin in utero are theoretically possible. The present results illustrate that such prenatal cross-fostering studies in humans are feasible and informative for disentangling the origins of health and behavior. Many behaviors, physiological processes, and common disorders (e.g., metabolic functioning, anxiety, osteoporosis, blood pressure) (30–33) are thought to originate in early life, and therefore this unusual experimental design is likely to be applicable to many scientific specialties.

Materials and Methods

Sample. Families who had a live birth between 1994 and 2002 (children aged 4–10 years), after successful IVF treatment from any of the conception groups described, were recruited from 19 U.K. clinics and 1 U.S. clinic. Gamete donors were unrelated to either parent. All initial contact was made through clinic staff. Data were collected through postal questionnaires and, where consent was provided, review of antenatal records. Wales Multi Centre Research Ethics Committee reviewed and approved the study. For data to be included in this report, we required a reply from the mother who reported on prenatal complications. Questionnaire data from 779 families were available: 387 homologous IVF (parents own gametes used), 187 IVF with sperm donation, 170 IVF with oocyte donation, and 35 IVF with embryo donation. The majority of women agreed for their antenatal records to be reviewed (77%). Antenatal record data were available from 483 of the 539 women who returned consent forms (90%).

Measures of Antenatal Predictor Variables. Smoking in pregnancy. Mothers reported on smoking during pregnancy and the number of cigarettes smoked per day (<10, 10–20, and >20). If either the mother report or antenatal records endorsed smoking in pregnancy (yes/no), the individual was assigned as a prenatal smoker. Previous work has shown that there is good agreement (κ=0.806) between the 2 data sources in this sample (34). The rate of smoking during pregnancy was 6%. There was no difference in the number of cigarettes smoked per day in the related and unrelated groups (χ² = 1.29, df = 2, P = 0.525). There was no association with smoking and drinking alcohol during pregnancy (χ² = 0.17, df = 1, P = 0.679) or the frequency of medication use in pregnancy (χ² = 0.45, df = 3, P = 0.929).

Outcome variables. Birth weight. Agreement between maternal reports and records for birth weight was nearly perfect (r = 0.985, P = 0.001), therefore maternal reports were used. Birth weight for singleton and multiple births was consistent with population norms (22).

Gestational age. Mothers reported during which week of pregnancy their child was born. Agreement with information in antenatal records was excellent (r = 0.959, P = 0.001).

Child antisocial behavior. Mothers and fathers completed the Strengths and Difficulties Questionnaire (35) about their child’s behavior. Antisocial behavior was assessed with 5 items (temper tantrums, fighting/bullying, disobedience, lying/cheating, and stealing). If either the mother or the father endorsed a symptom, it was counted as present. The average child antisocial behavior score in this sample (Table 1) was consistent with the general population norm of 1.67, SD = 1.7 (www.SDQinfo.com/bb1.html).

Statistical Analysis. Analysis of variance was used to test separately in the related and unrelated groups for associations between prenatal smoking and offspring outcomes. All tests were two-tailed. For ease of interpretation, birth weight and antisocial behavior were standardized, so that the population mean was equal to zero and the standard deviation was equal to one, and these standardized scores were analyzed. Partial χ² values are presented as a measure of effect size. The pattern of results also replicated when ordinary least regression was used (results available from F.R.).

Covariates. A number of covariates were included in each analysis. Child gender, multiple birth, and maternal height were included as covariates in analyses of birth weight because they have been shown to influence birth weight (36). Maternal education was additionally included as a covariate, because mothers who smoked during pregnancy had less formal education, than women who did not smoke during pregnancy (t = 3.50, df = 792, P = 0.001). Smokers reported higher levels of stress during the third trimester of pregnancy (t = 2.30, df = 755, P = 0.022), and late gestational stress has been reported to reduce birth weight (38). Therefore, we included late pregnancy stress as a covariate. Stress was measured by maternal report on an 11-point Likert scale and shows excellent test–retest reliability in this sample (intraclass correlation = 0.71). Finally, gestational age was included as a covariate to test whether smoking in pregnancy was associated with reductions in birth weight independently of gestational age.
Child gender, multiple birth, maternal education, maternal antisocial behavior, gestational stress, and maternal age at birth of child were included as covariates in analyses of antisocial behavior, because these factors have been shown to influence rates of antisocial behavior (39).

Analyses examining birth weight as an outcome were repeated, restricting the sample to singleton births. To test for gender differences, analyses were run separately for boys and girls (results available from F.R.).

When associations between prenatal smoking and offspring outcome were detected in the related or unrelated offspring separately, interactions were tested by using an ANOVA. The whole sample was analyzed with the interaction term between prenatal smoking and relatedness group and the main effect of prenatal adversity plus covariates (as described above). This interaction term therefore measured the difference in the effect of prenatal smoking on child outcome between the 2 offspring groups (related, unrelated). A significant interaction indicates that the association between prenatal adversity and child outcome differs significantly according to offspring group. Therefore, a significant interaction is expected when inherited factors explain the association between prenatal adversity and child outcome.

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