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Spontaneous Fetal Behavior After Maternal Exposure to Ethanol

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SMOTHERMAN, W P , K S WOODRUFF, S R ROBINSON, C DEL REAL, S BARRON AND E P RILEY *Spontaneous fetal behavior after maternal exposure to ethanol* PHARMACOL BIOCHEM BEHAV **24**(2) 165–170, 1986 —Acute and chronic intubations of ethanol to pregnant rats produced changes in spontaneous fetal behavior four hours later Fetuses from mothers in intermediate alcohol groups (4 and 6 g/kg) were substantially less active than controls (0 g/kg), but fetuses from low (2 g/kg) and high (8 g/kg) alcohol groups showed little indication of behavioral suppression Circulating levels of alcohol in maternal blood, fetal homogenate and amniotic fluid at the time fetuses were observed confirmed that fetuses were exposed to alcohol in utero, but the measured concentrations of alcohol were not predictive of fetal activity We suggest that some of the developmental consequences of Fetal Alcohol Syndrome may be the consequence of fetal inactivity induced by alcohol in utero

Fetal behavior Alcohol Fetal Alcohol Syndrome Teratogen Pregnancy Dysmorphogenesis Behavioral pathogenesis

ATTEMPTS to understand the etiology of Fetal Alcohol Syndrome in humans have emphasized the use of animal models [2, 3, 21] Exclusively, studies of the effects of prenatal alcohol exposure in animals have investigated the postnatal characteristics of offspring whose mothers had been administered alcohol during pregnancy Many of these studies have successfully identified behavioral effects of alcohol ranging from age-dependent hyperactivity to a variety of learning deficits [2,3] Although the origin of these behavioral changes is rooted in the prenatal environment, relatively little is known about how fetuses respond to alcohol in utero

Recently, techniques have been perfected that enable the direct observation of the behavior of rat fetuses in utero These techniques have permitted characterization of the ontogeny of spontaneous fetal movement [19], the response of fetuses to novel, familiar and aversive chemosensory stimuli in utero, and the ability of fetuses to form learned associations in utero [20] The findings of these and earlier investigations describe the rat fetus as active during the last third of gestation, sensitive to changes in its intrauterine environment, and integrally involved in its own development With the advent of this methodology, it is now possible to directly assess the effect of prenatal alcohol exposure on the spontaneous behavior of the rat fetus

In this study, we report that 19-day-old rat fetuses exhibited altered behavior in the presence of alcohol in utero However, the observed behavioral effect was not clearly related to alcohol content in maternal blood, fetal homogenate or amniotic fluid at the time of observation, four hours after maternal intubation

METHOD

Subjects and Housing

Adult female Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were maintained under conditions of constant room temperature (22°C), regulated light cycle (12 hours light, 12 hours dark, lights on at 0700), and continuous access to food and water Females were timed-mated with Long-Evans males, this cross produces large litters of vigorous offspring ideally suited to prenatal study [18,20] Vaginal smears were taken over the period of breeding to identify the beginning of gestation (the first sperm-bearing smear was designated Day 0)

Alcohol Intubation

In both experiments of this study, each subject female was selected late in gestation, food-deprived for four hours, and orally intubated with a solution of ethanol (in saline) or 0 9% saline as a control In Experiment 1, each female was

intubated once, on Day 19 of gestation The volume of ethanol intubated was adjusted to the rat's weight to provide five treatment groups with varying levels of ethanol administration (N=6 rats per group) 0 (the saline control), 2, 4, 6 and 8 g ethanol per kg body weight To manage the volume of ethanol intubated, rats in the 2 and 4 g/kg groups received a 20% w/v solution of ethanol in saline, whereas rats in the 6 and 8 g/kg groups received a 30% w/r solution of ethanol in saline Mean volumes (with range) intubated were 0 g/kg 6 8 ml (6 2–7 4), 2 g/kg 3 4 ml (3 2–3 6), 4 g/kg 6 6 ml (6 2–7 2), 6 g/kg 7 5 ml (7 2–8 0), 8 g/kg 9 2 ml (8 1–9 7) In Experiment 2, each female was orally intubated five times at 24-hr intervals, on Days 15 through 19 of gestation (N=6 rats per group) Only one dosage of alcohol was used in Experiment 2, 4 g/kg (20% w/v solution), with control animals receiving a 0 9% saline intubation In both experiments, on the day of behavioral observation—Day 19 of gestation—a delay of four hours elapsed before intubated females were surgically prepared for observation of fetuses The four hour delay was selected in conjunction with the intubation dosages to ensure that fetuses would be observed under diverse conditions of alcohol exposure

Surgical Preparation of Pregnant Females

Under ether anesthesia, a chemomyelotomy was performed to chemically transect the rat's spinal cord at the T12-L1 level This procedure produces an irreversible spinal block at the site of injection, thereby reducing afferent stimuli from the lower body without the use of long-lasting general anesthesia [16,19] The duration of ether anesthesia was very brief (generally less than 60 sec), and did not systematically vary among treatment groups The prepared female was secured in a holding apparatus and her abdomen and hindlimbs immersed in a bath of isotonic saline (Locke's solution) maintained at 37 5°C The rat's uterus was externalized through a midline laparotomy and allowed to float just beneath the surface of the bath A period of 15–20 min elapsed between the time of surgery and observation to allow females and fetuses to fully recover from the effects of ether anesthesia [10]

Observation Protocol

Two subject fetuses from each female were selected for behavioral observation, one from the ovarian end of each uterine horn Selection of fetuses from this standardized uterine position promotes fetal viability and ease of viewing throughout an observation period At the beginning of the first observation period one subject fetus was delivered from the uterus into the bath, preserving the attachment of the placenta to the uterine wall An observer then watched the spontaneous behavior of this fetus for 10 min, calling the events to a second person who maintained a continuous sequential record of observed behavior, elapsed time, activity of the prepared female, and bath temperature Following the first observation period, the second subject fetus was delivered from the uterus and observed for a period of 10 min All fetuses were observed by the same person, who was blind to the treatment administered to the mothers In previous experiments employing the same observational procedures, the process of recognizing and categorizing fetal behavior was found to be highly reliable [19,20] Observations of subject fetuses were carried out between 1100 and 1700 hours

Six basic categories of fetal behavior were distinguished

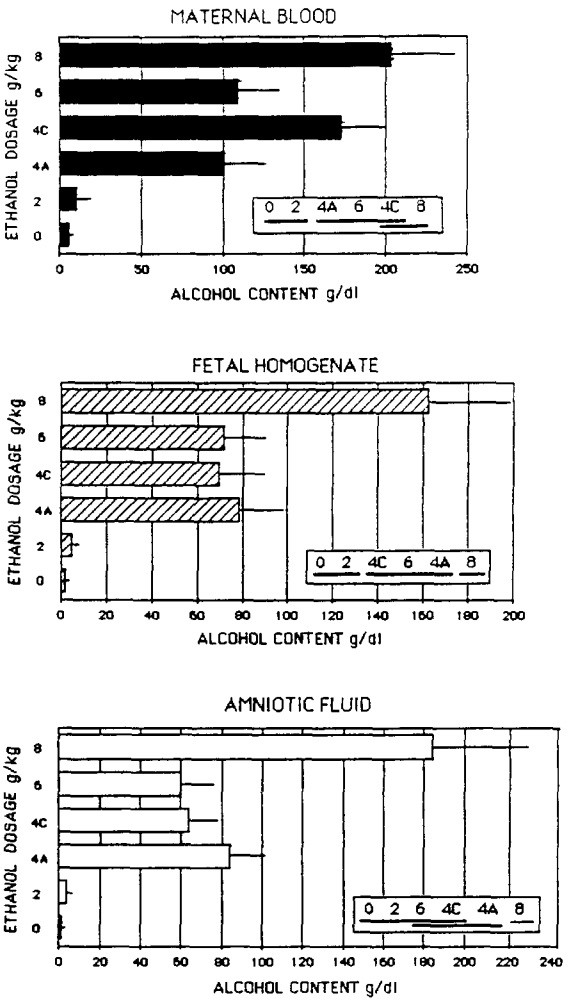

FIG 1 Assayed alcohol content (g/dl) of maternal blood, fetal homogenate and amniotic fluid for rats intubated with 0, 2, 4, 6, 8 g/kg ethanol (all acute) or 4 g/kg (chronic administration) Bars indicate mean values, horizontal lines show S E M Patterns of significant difference indicated by post-hoc tests are depicted in inset boxes groups underscored by the same line did not differ

[20] These categories comprised individual movements of Head, Mouth, Forelimbs, Hindlimbs, Trunk (whole body movement) and Twitch (a lateral spasm) To aid description of overall fetal activity, three additional categories were derived from the six basic behavioral patterns Complex Movement included all instances of activity that comprised two or more simultaneous fetal behaviors Whole Activity was the number of times a fetus was recorded as active (i e , each simple or complex movement was scored as one event), Component Activity was the sum of all basic behavioral movements of a fetus (i e , each simple movement was scored as one event, each behavioral component of a complex movement was scored as a separate event) In previous studies of fetal behavior, we have found no evidence that the sex of the fetus affects behavioral responses, the sex of subject fetuses therefore was not considered in the analyses Frequency counts in the six basic behavioral categories and three derived categories reflecting overall activity were analyzed by ANOVA to evaluate behavioral changes under

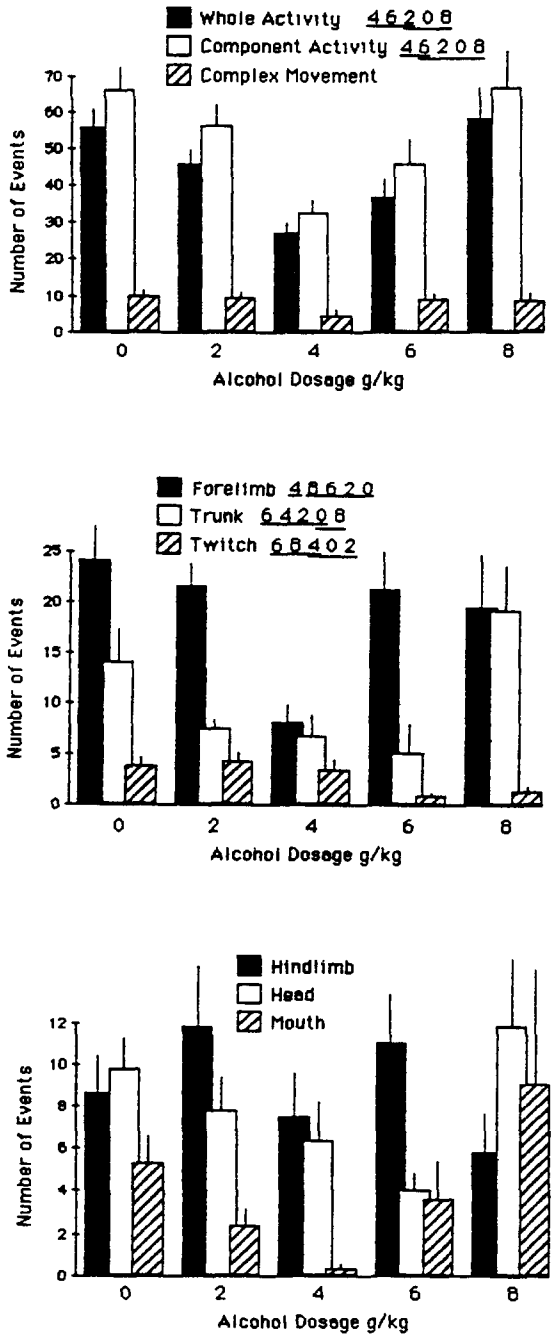

FIG 2 Number of behavioral events for nine categories of spontaneous fetal behavior following acute alcohol intubation (Top) frequencies of Whole Activity, Component Activity and Complex Movement, (Middle) frequencies of Forelimb Trunk and Twitch (Bottom) frequencies of Hindlimb, Head and Mouth Bars represent mean frequencies (±S E M) at five levels of acute intubation 0, 2, 4 6 and 8 g/kg ethanol Groups underscored by the same line did not differ

different conditions of acute (Experiment 1) or chronic (Experiment 2) alcohol administration

Collection of Samples for Alcohol Determination

Immediately following observation of the two subject fetuses (4 5 hr after intubation in both experiments), the prepared female and remaining fetuses provided samples of maternal blood, fetal homogenate, and amniotic fluid for assay of alcohol content Blood was collected from mothers by cardiac puncture and transferred to heparinized tubes Amniotic fluid and fetal homogenate were collected from randomly selected fetuses within both uterine horns (range=5–8 per mother), excluding the two subject fetuses Samples of fetal homogenate comprised fetal tissues, placenta and amniotic fluid All samples were immediately frozen until assay Alcohol content in all samples of maternal blood, amniotic fluid and fetal homogenate was assayed by a quantitative enzymatic (alcohol dehydrogenase and nicotinamide adenine dinucleotide) procedure (Sigma Technical Bulletin no 332, Sigma Chemical Company)

Morphological Measurement

In addition to samples for alcohol determination, females from Experiment 2 provided sample fetuses for measurement of fetal body weight, umbilical cord length, or amniotic fluid volume Fetuses were selected from among the remaining non-observed fetuses, removed from the uterus, and placed on a paper towel soaked with isotonic saline Body weights were taken from fetuses that were removed from the amnion, patted dry with a towel, and separated from umbilical cord and placenta Umbilical cord length was measured with a compass and millimeter ruler from the placental plate to the abdominal wall Uniform traction was applied to the cord by gently pulling the fetus in a horizontal direction This procedure produced measurements that were replicable for a given fetus and accurate to about 0 5 mm Amniotic fluid was sampled by puncturing the intact amnion and drawing the fluid into a 1 0 ml syringe, providing volume measurements that were replicable and accurate to 0 01 ml While the fluid sampled in this manner is primarily of amniotic origin, it contains small amounts of exocoel fluid [4]

RESULTS

Alcohol Content of Maternal Blood, Fetal Homogenate and Amniotic Fluid

To confirm that fetuses were exposed to alcohol in utero, samples of maternal blood, fetal homogenate and amniotic fluid were assayed for alcohol content A set of preliminary analyses of the fetal homogenate and amniotic fluid assay data indicated that dosage effects were confounded with litter effects [1,7] For these two measures, samples from multiple fetuses within the same mother were combined to provide a litter mean, which was used in all subsequent analyses

A series of one-way ANOVAs indicated significant effects of dosage in maternal blood, $F(5,36)=14\ 0$, $p<0\ 001$, fetal homogenate, $F(5,30)=9\ 4$, $p<0\ 001$, and amniotic fluid, $F(5,30)=11\ 4$, $p<0\ 001$ Post-hoc tests by the method of Newman-Keuls [24] indicated that fetuses in different dosage groups were exposed to three discriminable levels of alcohol 4 5 hours after intubation negligible (0 and 2 g/kg), intermediate (4 and 6 g/kg acute, 4 g/kg chronic), and high (8 g/kg) These data and patterns of difference betwen dosages are depicted in Fig 1 A second series of one-way ANOVAs was performed to compare alcohol content in the three sampled reservoirs Acute administration resulted in an equal distribution of alcohol in maternal blood, fetal homogenate and amniotic fluid at all intubation dosages Chronic administration, however, produced significantly higher alcohol

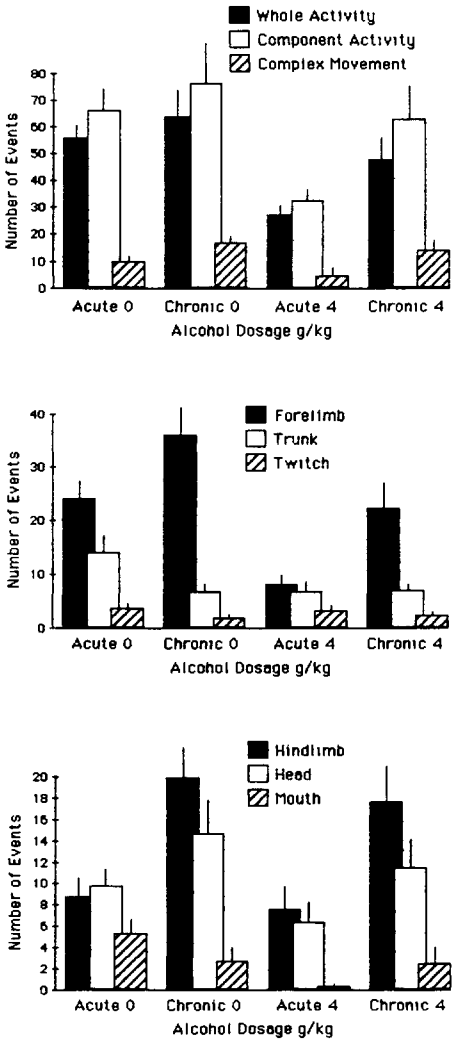

FIG 3 Number of behavioral events for nine categories of spontaneous fetal behavior following acute or chronic intubation of saline (0) or alcohol (4 g/kg) (Top) frequencies of Whole Activity, Component Activity and Complex Movement, (Middle) frequencies of Forelimb, Trunk and Twitch, (Bottom) frequencies of Hindlimb, Head and Mouth Bars represent mean frequencies (±S E M)

levels in maternal blood than in fetal homogenate or amniotic fluid, $F(2,15)=10\ 7$, $p<0\ 01$ In summary, the assay data provide independent confirmation that the intubation procedures were effective in exposing fetuses to different levels of alcohol in utero

Behavioral Results

In studies where behavioral data are collected from multiple offspring of the same mother, treatment effects may be confounded with litter effects [5] Preliminary analyses of data in this study revealed no significant litter effects, however, and behavioral data from all subject fetuses were considered independent and analyzed by one-way ANOVA In the following summary, all comments regarding differences between groups are based upon post-hoc comparisons following the technique of Newman-Keuls ([24] p 191)

Experiment 1 Acute Administration of Alcohol

An effect of alcohol on fetal behavior was evident for two measures of general activity and for three specific categories of fetal movement (Fig 2) The number of times a fetus was recorded as active (Whole Activity) showed a significant decrease in the 4 and 6 g/kg groups relative to 0, 2 and 8 g/kg groups, $F(4,55)=5\ 8$, $p<0\ 001$ The magnitude of this overall behavioral suppression was dramatic movement by fetuses in the 4 g/kg group was reduced by over half (51% fewer movements than controls) and in the 6 g/kg group by a third (34% fewer movements) Similarly, the number of individual body-part movements (Component Activity) was significantly reduced in the 4 g/kg group relative to all other dosage groups, $F(4,55)=4\ 6$, $p<0\ 01$ Among specific behavioral patterns affected by alcohol, Trunk movements (curling and stretching motions) were less frequent in 2, 4 and 6 g/kg groups than in 8 g/kg, but none of the alcohol dosage groups differed from control levels, $F(4,55)=4\ 2$, $p<0\ 01$ Forelimb movements were reduced in the 4 g/kg group, $F(4,55)=3\ 2$, $p<0\ 05$, but no differences were evident among 0, 2, 6 and 8 g/kg groups Twitches were less frequent in 6 and 8 g/kg groups than in 0, 2 and 4 g/kg groups, $F(4,55)=4\ 3$, $p<0\ 01$ The remaining three specific patterns of fetal movement did not evince any effect of alcohol

Experiment 2 Chronic Administration of Alcohol

Behavioral data from the 0 and 4 g/kg acute intubation groups and the 0 and 4 g/kg chronic intubation groups were compared in a two-way ANOVA to assess the effects of alcohol dosage and intubation pattern on fetal activity Because the acute intubation data were involved in a second analysis, a more conservative alpha level (0 01) was used to judge significance in these tests

A main effect of alcohol dosage (0 vs 4 g/kg) was evident for both measures of general activity, Whole Activity $F(1,44)=16\ 5$, $p<0\ 01$, Component Activity $F(1,44)=8\ 5$, $p<0\ 01$ In both cases, overall activity was suppressed in alcohol intubation groups (Fig 3) This pattern of reduced movement was also apparent for Forelimb movements, $F(1,44)=13\ 7$, $p<0\ 01$, which occurred only half as often in alcohol-exposed fetuses

The pattern of intubation (acute vs chronic) affected the frequency of Complex Movement by fetuses, $F(1,44)=13\ 6$, $p<0\ 01$, with chronic-exposed subjects exhibiting more than twice the activity of acute-exposed subjects Among specific patterns of fetal behavior, Forelimb movements, $F(1,44)=10\ 4$, $p<0\ 01$, and Hindlimb movements, $F(1,44)=17\ 3$, $p<0\ 01$, were both much less frequent in acute intubation groups than in chronic intubation groups The interaction of intubation pattern by alcohol dosage was not significant for any general or specific category of fetal behavior Thus, there was no evidence to indicate that fetuses exposed to alcohol responded differently under acute or chronic administration

Morphological Measurements of Fetuses Chronically Exposed to Alcohol

Fetuses that had been exposed to alcohol for five days in Experiment 2 were also examined to provide morphological data on fetal body weight, unbilical cord length, and amniotic fluid volume No significant differences were apparent between control and chronic alcohol groups for either body weight (0 g/kg 2 81±0 18 g, 4 g/kg 2 75±0 21 g) or umbilical

cord length (0 g/kg 25 3±2 1 mm, 4 g/kg 25 3±2 3 mm) These measurements agree closely with data for unmanipulated 19-day-old fetuses in our laboratory (body weight=2 71±0 04 g, cord length=25 3±0.43 mm, unpublished data), suggesting that the intubation procedures did not impair normal development However, alcohol-exposed fetuses did show significantly reduced amniotic fluid volume relative to controls (0 g/kg 0 45±0 07 ml, 4 g/kg 0 36±0 04 ml, $t(10)=2$ 73, $p<0$ 05, unmanipulated Day 19 fluid volume=0 51±0 01 ml)

DISCUSSION

The results of the behavioral observations clearly demonstrate that spontaneous behavior of rat fetuses in utero can be affected by maternal intubation with alcohol A one-time intubation of 4 g/kg ethanol resulted in a 51% reduction in overall activity, including a specific suppression of Forelimb movement The suppression of the latter behavioral category is of particular interest because Forelimb movements constitute the most frequent activity of unmanipulated fetuses on Day 19 of gestation [19,20] and are especially sensitive to perturbations in the uterine environment, such as oxygen deficiency [23] This finding provides the most direct evidence to date that maternal ingestion of alcohol during pregnancy affects the spontaneous behavior of fetuses It is also consistent with the indirect monitoring results of McLeod *et al* [11], who demonstrated a suppression of "breathing" movements in human fetuses following an acute ingestion of alcohol by the mother

The pattern of behavioral suppression across the various dosage groups suggests that fetal activity cannot be predicted from the current levels of alcohol in maternal blood, fetal homogenate or amniotic fluid This conclusion is most striking for fetuses in the 8 g/kg group With the single exception of Twitch movements, fetuses exposed to 8 g/kg ethanol showed no reduction in activity relative to controls Yet these fetuses experienced the highest levels of alcohol 4 hours after intubation It is important to note that the lack of behavioral suppression in the highest alcohol group is specific to the time of observation employed in this study A time-course examination of fetal responses to alcohol could help to elucidate the interaction of dosage and time in the control of fetal behavior For example, fetal responses may be different when blood alcohol levels are rising, immediately following intubation, than when alcohol levels are falling several hours later At present, however, circulating levels of alcohol in mother and fetus are unreliable as predictors of fetal response

Behavioral suppressions were evident with both the first exposure to alcohol (acute group) and the fifth (chronic) From this evidence it could be argued that fetal responses to alcohol show no evidence of adaptation or behavioral tolerance However, this interpretation is qualified by indications that the procedures involved in chronic intubation affected fetal behavior fetuses were more active in chronic groups regardless of whether alcohol or saline was intubated

Alcohol can suppress fetal behavior This is potentially important, because studies of human fetuses in clinical situations and controlled experiments with rodent fetuses have revealed how behavioral suppression in utero can have profound effects on morphological development Fetal inactivity, resulting from congenital myopathy, neural dysfunction, oligohydramnios, or experimental curare administration, causes fetuses to develop physical anomalies, such as joint contractures, microstomia and micrognathia, redundant skin folds, pulmonary hypoplasia and growth deficiency [12, 14, 15] In this regard, it is noteworthy that the chronic alcohol group exhibited significantly reduced amniotic fluid volume (oligohydramnios), which may also contribute to disruptive mechanical dysmorphogenesis [8, 17, 22] The involvement of fetal inactivity and abnormal morphological and behavioral development associated with prenatal alcohol exposure suggests that altered fetal behavior also may be a contributing factor in the etiology of Fetal Alcohol Syndrome [6]

Behavior is not a trivial aspect of fetal life, coordinated movement plays a necessary role in the fetus's normal physical and behavioral development [9,13] Therefore, any substance or external influence that alters fetal behavior over an extended time could be viewed as a potential teratogen Conversely, the direct observation or indirect monitoring of fetal behavior in utero may provide a powerful early indicator of behavioral and physical abnormalities that may persist and develop into postnatal syndromes

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