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Teratologic Evaluation of Rats Prenatally Exposed to Pulsed-Wave Ultrasound

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ABSTRACT While there are no known risks associated with diagnostic ultrasound, uncertainty about the safety of prenatal ultrasound exposure remains. The purpose of the present experiment was to evaluate the teratogenic potential of pulsed-wave (pw) ultrasound in rats, in the absence of maternal anesthesia or restraint. Pregnant CD rats, trained to remain immobile in a water-filled ultrasound exposure tank, were scanned with 3-MHz pw ultrasound at levels of 0, 2, 20 or 30 W/cm^2 I_{SPTA} (spatial peak, temporal average intensity) on gestational days 4–19 for approximately 10 min/day. Examination of fetuses on E20 revealed no increase in skeletal or visceral malformations in groups exposed to pw ultrasound in utero. The number of implantations/dam was significantly increased in all pw ultrasound exposure groups compared to sham-exposed animals and, in a possibly related finding, resorptions were increased in the two highest exposure groups. Although significantly increased compared to controls, resorption frequencies in these groups were low (<10%). No exposure-related changes in fetal weights were observed in offspring of rats scanned with pw ultrasound during gestation. The results indicate that, under the conditions described, no overt embryotoxicity is associated with gestational exposure to pw ultrasound intensities of up to 30 W/cm^2.

Sonographic examination has become a routine obstetrical procedure in many countries, including the United States. The widespread use of diagnostic ultrasound in pregnancy reflects a consensus among clinicians that the technique is safe as well as beneficial, and this view is generally supported by the epidemiological literature (NIH, '84; Brent et al., '91). No evidence of adverse effects has emerged from the extensive clinical experience with ultrasound over more than 25 years. But, while epidemiological studies of fetal ultrasound exposure have been mostly reassuring, recognition of the methodological difficulties and inherent limitations of such studies in detecting subtle effects or low levels of risk has warranted continued caution (Ziskin and Petitti, '88). In addition, two recent developments in clinical practice, the use of diagnostic ultrasound devices with higher acoustic output intensities and the more frequent use of ultrasound during the preconception and early gestational periods, have prompted a reappraisal of possible reproductive risks (Miller, '90; Martin et al., '91).

Experimental efforts to delineate the bioeffects of ultrasound on in utero development have been inconclusive (Brent et al., '91; Carstensen and Gates, '85; O'Brien, '85). Some studies have reported increased malformation rates (Mannor et al., '72; Shoji et al., '75; Sikov and Hildebrand, '76; Pizzarello et al., '78; Stolzenberg et al., '80; Saravazyan et al., '82; Takabayashi et al., '85), while others have found no such effects (McClain et al., '72; O'Brien et al., '82; Child et al., '84, '88; Kimmel et al., '83, '89). Effects of ultrasound exposure on fetal body weight have also been reported in some studies (Pizzarello et al., '78; Stolzenberg et al., '80; O'Brien, '83; Tarantal and Hendrickx, '89a), but not in others (Child et al., '84, '89; Kimmel et al., '83, '89). Part of the difficulty in assessing the developmental effects of ultrasound based on animal data lies in the variability of the experimental conditions, exposure parameters, and endpoints used. Where biological alterations have been found their significance is often unclear because appropriate dose–effect relationships have not been established. There are also a number of other uncertainties with respect to the extrapolation of experimental animal data to humans, such as those concerning possible differences in attenuation, target size, species sensitivity, and heating effects (NIH, '84).

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A limitation common to most animal studies of ultrasound developmental effects involves the use of anesthesia or restraint during insonation. These factors could potentially confound the experimental results, since both are themselves associated with developmental toxicity (Mazze et al., '85; Weinstock et al., '88). Furthermore, the circulatory effects of anesthesia could alter the response to ultrasound-induced hyperthermia. In order to eliminate these potential confounders, we developed an ultrasound exposure system for insonating rats that have been trained to remain immobile (Vorhees et al., '91). This system was previously used to assess the effects of prenatal exposure to continuous-wave ultrasound on growth and development in rats. The present study extends this approach by examining the developmental effects of in utero exposure of rats to pulsed-wave (pw) ultrasound using the conditioned immobility procedure.

MATERIAL AND METHODS

Animals

Subjects were nulliparous female Sprague-Dawley CD (VAF) rats (Charles River, Portage, MI), housed according to the guidelines of the American Association for the Accreditation of Laboratory Animal Care. Prior to placing females with males, immobility training was conducted in a water-filled chamber approximately the same size as that of the confinement chamber in the ultrasound exposure tank. Each female received two consecutive days of 15 min/day followed by 2 days of 10 min/day confinement in the training tank. This repeated confinement induced conditioned immobility, i.e., an attenuation of efforts to escape. Females were housed with males on the day following the last training session. Discovery of a vaginal plug was considered embryonic (E) day 0.

On E0, dams were assigned to one of four treatment groups for pulsed-wave (pw) ultrasound exposure on a weight-matched basis and encoded on the exposimetry computer so that experimenters were blind to treatment group assignment. At least 10 dams were assigned to each group. On E3, each dam received refresher immobility training (15 min) in the exposure tank. After removal from the vessel, the abdomen of each rat was depilated.

Exposure system

For detailed descriptions of the ultrasound exposimetry system that was developed specifically for these experiments, see Smith et al. ('90) and Vorhees et al. ('91). Briefly, the exposure system consisted of a water-filled, rectangular container constructed of acrylic, with a partially focused 4.6-cm diameter, 3 MHz, PZT-4 crystal mounted in a moveable transducer assembly platform approximately 30 cm below the water surface. Pregnant rats that had been trained to remain immobile were placed in an inner confinement chamber (10 × 15 cm) directly above the ultrasound beam (Fig. 1). The ultrasound beam was calibrated under free-field conditions with a calibrated membrane hydrophone (Marconi). For a stationary beam, at the focus that would be a location within the floating rat, the free-field 90% and 50% intensity beam widths were 1.6 cm and 3.2 cm, respectively. At the focus the four pw values of the spatial peak, temporal average intensity (I_{SPTA}) used were 0, 2.0, 20, and 30 W/cm^2, as calculated from the measured instantaneous pressures, all at a spatial peak, pulse average (I_{SPPA}) value of 40 W/cm^2.

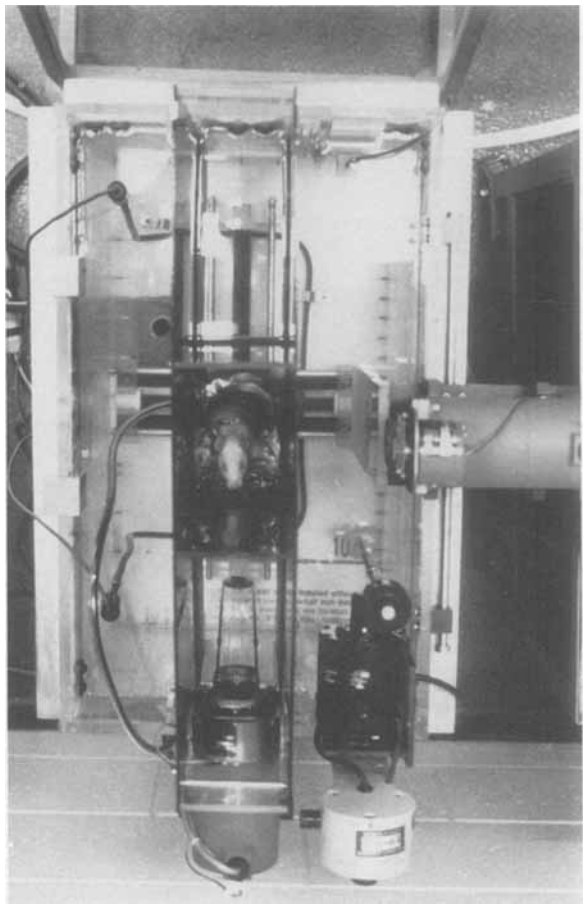

Fig. 1. Top view of the ultrasound exposure system showing trained rat in the 10 × 15-cm confinement chamber. Note the movable transducer assembly situated below the floating rat.

A modification of an approach described by O'Brien et al. ('82) was used to provide uniform ultrasound exposure to the floating rat's abdominal surface, which was estimated to be approximately 7 (width at the widest point) × 8 cm (xiphoid process to the prepuce) on E17. A raster scan pattern of the moveable transducer assembly was set at 8 × 13.5 cm, or approximately 1 cm inside each wall of the confinement chamber, so that the entire enclosure was insonated. The raster

scan sequence consisted of 16- 8 cm rasters separated by 1 cm. Each rat received one raster scan per day on E4–19 for a total of 16 insonation exposures, each lasting 10 min, with 2 min of transducer positioning time at the beginning of the scanning pattern for a total of 12 min per session. Based on the raster scan pattern and transducer speed, the time that a specific body site was within the 90% intensity beam width during one scanning session was determined to be 8–12 sec. For the 50% intensity beam width the exposure time ranged between 41 and 46.5 sec.

The exposure tank was refilled daily with fresh deionized water and allowed to degas overnight. Water temperature was maintained at 35°C by a Yellow Springs Proportional Temperature Controller (model 72).

Teratological procedures

On E20, all dams were euthanized with CO_2 and a laparotomy was performed. The uterine horns were exteriorized and the contents examined for implantation sites. Each fetus' position was noted; it was then removed, sexed, weighed, and examined externally. Every other fetus in order was placed in Bouin's solution for at least 2 weeks, sliced freehand, and examined for cephalic and visceral defects by the method of Wilson ('65). The remaining fetuses were immersed in hot water to facilitate skin removal, placed in 95% ethanol and later eviscerated, cleared in potassium hydroxide, and double stained with alizarin red S and Alcian blue for skeletal examination.

Statistical procedures

Continuous data, such as body weight, were analyzed using fixed-effect factorial analysis of variance, with litter as the unit of analysis. Frequency data, such as percentage malformed offspring and mortality, were analyzed by Fishers's test for uncorrelated proportions. For all ANOVAs, a posteriori group comparisons were conducted using Duncan's multiple range test. For analysis of proportion malformed per litter, the data were transformed according to the procedure of Kirk (1968) because of the high frequency of zero values, and analyzed by ANOVA.

RESULTS

No treatment-related effects on maternal body weight during gestation were found in an analysis of variance (ANOVA) with repeated measures for day. Reproductive and fetal outcome data for pw ultrasound exposure are summarized in Table 1. No significant preimplantation loss was associated with pw exposure. An ANOVA performed on implantation number revealed a significant group effect ($P<0.002$), with all three pw exposure groups having an increased number of implantations compared to controls. Fisher's tests showed significant increases in the percentage resorbed in the two highest exposure groups (20 and 30 W/cm^2) compared to controls (both comparisons significant at $P<0.05$), but when the resorption data were analyzed by litter using an ANOVA, no significant treatment group differences were found. There was a trend for a group effect on number of live fetuses per litter ($P<0.07$). The simple effect analysis showed live fetuses per litter was significantly increased in the high exposure group compared to the control group ($P<0.05$).

No significant treatment group differences in total, skeletal, or visceral malformations were found, either in the aggregate or by litter analysis. The abnormalities seen were: one fetus with rib defects each in the 2.0- and 20-W/cm^2 groups, one fetus with a ventricular septal defect and one with bilateral hydroenphrosis in the control group, one fetus with renal agenesis in the 20-W/cm^2 group and one fetus with an enlarged right ventricle in the 30-W/cm^2 group. An ANOVA performed on fetal body weights revealed no treatment-related effects.

DISCUSSION

We employed a conditioned immobilization procedure to examine the developmental effects of gestational pw ultrasound exposure in rats without the use of anesthesia or forced restraint. The data showed that such insonation produced no adverse effects on maternal body weight or pre-implantation embryonic loss, and no increase in fetal malformations. There was an increased resorption rate in the two highest pw exposure groups when these were compared individually with the control group. This effect may be related to the increased number of implantations noted in these groups. The effect on implantations did not appear to be dose dependent, since implantations were also increased in the group exposed to the lowest ultrasound intensity, which had a nonsignificant increase in resorptions compared to controls. There was also a trend for more live fetuses in the ultrasound exposed groups, with the highest exposure group significantly different from controls. But, in a follow-up study (unpublished) conducted to evaluate long-term behavioral effects of prenatal pw ultrasound exposure, using the same exposure conditions and intensity levels, there was no difference in the number of live births among the four treatment groups. While resorption is an important parameter for assessing embryotoxicity, it is known to be highly variable, even within the same rat strain (Charles River, '88). The magnitude of the effect on resorption in the present study was small and probably within the range of normal biological variability. Finally, fetal weight, which is thought to be one of the most sensitive indicators of embryotoxicity, was not affected by in utero exposure to even high intensity pw ultrasound.

In addition to prenatal ultrasound-associated terato-

TABLE 1. Effects of pw ultrasound on embryonic development: effects of pw ultrasound[1] on reproductive outcome and embryonic development assessed on E20 in rat dams insonated on E4–19

Dependent variable	Group (W/cm^2) I_{SPTA}			
	0	2.0	20.0	30.0
No. sperm+females[2]	10	11	10	10
No. nonparturient dams[3]	0	2	0	0
No. implants/dam	13.8	16.7*	16.7*	17.6*
No. resorptions/tot. implants	2/138	7/150	12/167	12/176
% resorbed	1.4	4.0	7.2*	6.8*
No. live fetuses	136	143	155	164
No. live fetuses/dam	13.6	15.9	15.5	16.4*
Malformations (aggregate)				
No. (%) Total	2/136 (1.5)	1/143 (0.7)	2/155 (1.2)	1/164 (0.6)
No. (%) Skel.	0/65 (0)	1/69 (1.4)	1/75 (1.3)	0/79 (0)
No. (%) Visc.	2/71 (2.8)	0/74 (0)	1/80 (1.3)	1/85 (1.2)
Malformations (by litter)[4]				
Total	0.5 ± 0.04	0.4 ± 0.02	0.4 ± 0.04	0.4 ± 0.03
Skeletal	0.6 ± 0.03	0.6 ± 0.03	0.6 ± 0.04	0.6 ± 0.01
Visceral	0.6 ± 0.1	0.5 ± 0.02	0.6 ± 0.1	0.5 ± 0.04
Fetal body wt. by litter (g)[4]	3.54 ± 0.12	3.59 ± 0.13	3.55 ± 0.13	3.56 ± 0.05

[1]I_{SPPA}, 40 W/cm^2 for the three exposure groups.
[2]Number of dams assigned to each treatment group.
[3]All nonparturient dams were negative for implantations.
[4]Values represent the group mean ±SEM.
*Significantly different from control (P <0.05).

genicity and fetal weight reduction, possible effects of ultrasound on embryonic/fetal viability have been observed in several animal studies. McClain et al. ('72) reported small increases in the percentage of resorptions in rats exposed to diagnostic levels of cw ultrasound on several prenatal days. The differences did not reach the set significance level (P<0.02), however, and were not considered biologically important by the authors. In experiments by Shoji et al. ('72, '75) an increased incidence of prenatal death, which was accompanied by reduced fetal weights and increased malformations, was found in two inbred mouse strains exposed to low-intensity (40-mW/cm^2) cw ultrasound for 5 hr on day 8 or 9 of gestation. It has been pointed out that ultrasound-induced hyperthermia or maternal restraint stress associated with the prolonged exposures could have resulted in these observations (Lele, '75; O'Brien, '82). A study conducted by Stolzenberg et al. ('80) found increased resorptions, decreased numbers of viable fetuses, and decreased fetal weights in mice exposed to cw ultrasound (1 W/cm^2) for 400 sec on day 1 or day 13 of gestation. The authors concluded that a thermal mechanism was probably involved in the production of these effects. Pizarello et al. ('78) reported an increased resorption rate as well as decreased fetal weights in rats exposed to low intensity pw ultrasound during early gestation. These findings could not be replicated, however, even when higher intensities were used (Child et al., '84). Recently, Hande and Devi ('92) reported a small increase in the resorption rate and a reduction in fetal body weight in mice exposed to diagnostic pw ultrasound for 10 min on day 3.5 of gestation. All the aforementioned studies were potentially confounded by anesthesia and/or restraint.

In a previous study (Vorhees et al., '91) we found no evidence of embryotoxicity after prenatal exposure of rats to levels of cw ultrasound up to 30 W/cm^2, equivalent to the highest exposure in the present study in terms of spatial peak, temporal average intensity. But, cw and pw ultrasound have different physical properties, and there may be qualitative differences in the biological effects produced by pw as opposed to cw ultrasound (Sarvazyan et al., '82; Carstensen, '87). One of the mechanisms by which ultrasound can produce biological effects is heating (Nyborg, '85). For a thermal mechanism of action, which is thought to be important in many reported instances of ultrasound-induced embryotoxicity (Lele, '75), pw ultrasound would be expected to have the same biological effects as cw ultrasound at the same time-averaged intensity (NCRP, '83). However, there is evidence to suggest that temporal average intensity is not an appropriate basis for predicting some ultrasound bioeffects (Child et al., '81). In a review of clinical and experimental data, Stewart et al. ('85) concluded that the peak intensity as well as other pulse parameters such as pulse width and pulse repetition frequency may be important for the production of some biological effects. Although there have been few studies directly comparing cw and pw exposures, the authors stated that exposure to pw sources appeared to be associated with effects at lower temporal average intensity levels than for cw exposures. Studies of the response of *Drosophila* larvae to pw ultrasound showed that larval killing depended on the maximum intensity during the pulse rather than the

time-averaged intensity (Child et al., '81). The threshold for this effect, which is thought to represent an acoustic cavitation phenomenon, was determined to occur at a temporal peak intensity of 10–20 W/cm^2. The relevance of such effects for higher organisms has not been established. Results obtained by Takabayashi et al. ('85) from a dose–effect study in mice, however, suggested that pulse peak intensity and pulse width were the most important determinants of pw ultrasound-associated teratogenicity. In that study, exposure of two groups to equivalent average acoustic intensities of pw ultrasound resulted in an increased incidence of fetal malformations only in the group exposed to the higher peak intensity. Under their experimental conditions Takabayashi et al. also reported an apparent peak intensity threshold for pw ultrasound-association malformations in mice, which they estimated to be 60 W/cm^2. An independent attempt to replicate the findings from this study was unsuccessful (Child et al., '88).

While the temporal average intensity levels used in the present study were much higher than typical clinical exposure levels from pw imaging devices, the lowest intensity was within the upper limits of average intensities produced by some pulse Doppler devices in use today (Duck, '89). Since no clear effects were seen, even at the highest exposure levels where heating was expected to occur, this implies a large margin of safety for this type of exposure. However, if cavitation or other nonthermal mechanisms are considered, the pulse parameters may be of critical importance. The pulse average intensity of 40 W/cm^2 used for all three exposures is within the range for present commercial instrumentation. Some diagnostic pw devices have pulse intensities as high as 1,000 W/cm^2 (Miller, '91), however, and the approval process by FDA for diagnostic ultrasound devices allows for the pulse average intensity to be as high as 190 W/cm^2 (FDA, '85). The possibility that adverse effects on embryonic development could be produced by exposure to ultrasound with different pulse characteristics cannot be ruled out.

A variety of effects other than those which could have been detected in this study have been attributed to experimental prenatal ultrasound exposure. Many of these involve CNS development and include effects on brain morphometry (Norton et al., '90), myelination (Ellisman et al., '87), auditory evoked potentials (Moore et al., '85), neuromuscular maturation (Murai et al., '75a; Sikov et al., '77; Norton et al., '91), emotional behavior (Murai et al., '75b), and cognitive processes (Tarantal and Hendrickx, '89b). Since interpretation of many of these studies is hampered by the presence of confounding factors, as is true for the teratological studies, we are currently conducting experiments to assess the long-term behavioral consequences of prenatal ultrasound exposure in unanesthetized, unrestrained rats.

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