TERATOLOGY 44:667–674 (1991)

A Teratologic Evaluation of Continuous-Wave, Daily Ultrasound Exposure in Unanesthetized Pregnant Rats

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ABSTRACT Pregnant Sprague-Dawley rats were trained to remain immobile when placed in water in an ultrasound exposure tank and exposed to 0, 0.1, 2.0, or 30.0 W/cm^2 I_{SPTA} (spatial peak, temporal average), 3.0-MHz continuous wave (cw) ultrasound on embryonic (E) days 4–19 for approximately 15 min/day. On E20 fetuses were removed; weighed; examined for external, skeletal, and visceral malformations; and uteri were examined for resorptions. Analyses revealed no increase in pre-implantation loss and no effects on maternal body weight, food, or water consumption. No increase in skeletal or visceral malformations was found, in fact exposed groups had a lower incidence of defects than controls. A significant increase in resorptions in the lowest exposure group (0.1 W/cm^2) was obtained, but the effect was isolated, non-dose dependent and not credible as a treatment-related effect. No reduction in fetal weight was obtained, in fact the lowest (0.1-W/cm^2) and middle (2.0-W/cm^2) exposure level groups weighed slightly more than controls. The immobility procedure succeeded in avoiding anesthetization or forced restraint of the dams, thereby eliminating these factors as potential confounders. The results demonstrated that in unanesthetized, unrestrained rats in utero exposure to incident intensities of ultrasound of up to 30.0 W/cm^2 cw ultrasound (or estimated internal exposures of 4–21 W/cm^2, depending on body orientation to the incident beam) produced no evidence of embryotoxicity based on fetal necropsy data.

It has been estimated that >50% of all infants born in the United States and Europe receive intrauterine diagnostic ultrasound exposure (NIH, '84). Epidemiological investigations have not uncovered any clear evidence of insonation-associated teratogenicity, however, these investigations contain multiple methodological and theoretical limitations that make firm conclusions difficult (Ziskin and Petitti, '88). Efforts to clarify the bioeffects of ultrasound on in utero development using animals have produced mixed results. For malformations, some investigations have reported increased rates of defects (Mannor et al., '72; Shoji et al., '75; Sikov and Hildebrand, '76; Hara et al., '77; Pizzarello et al., '78; Stolzenberg et al., '80; Sarvazyan et al., '82; Takabayashi et al., '85), but others have found no such increase (Takeuchi et al., '70; McClain et al., '72; O'Brien et al., '82; Kim et al., '83; Barnett, '83; Child et al., '84, '88; Kimmel et al., '83, '89). Kimmel et al. ('89) noted that most of the positive studies have used insonation intensities and durations that induce some degree of hyperthermia and hyperthermia is teratogenic (e.g., Shiota, '88).

Effects other than malformations have

Received February 1, 1991; accepted August 27, 1991.

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also been reported in progeny exposed to ultrasound in utero including effects on brain morphometry (Rosenthal et al., '88; Norton et al., '90), myelination (Ellisman et al., '87), auditory evoked potentials (Moore et al., '85), reflexology (Murai et al., '75a; Sikov et al., '77; Norton et al., '91), vocalization (Murai et al., '75b), immunological development (Desai et al., '89a,b; Sosolik et al., '89), and cognitive processes (Tarantal and Hendrickx, '89b).

Another effect reported to be associated with in utero ultrasound exposure is fetal body weight reduction (Pizzarello et al., '78; Stolzenberg et al., '80; O'Brien, '83; Tarantal and Hendrickx, '89a). Here also the data are conflicting. Several studies have reported no reduction in fetal weight after ultrasound exposure (Child et al., '84, '89; Kimmel et al., '83, '89).

One characteristic common to all the studies cited above, except for the one primate experiment (Tarantal and Hendrickx, '89a,b), is that the animals were either anesthetized or forcibly restrained during insonation. However, some anesthetics (e.g., McColl et al., '67; Mazze et al., '84, '85) and restraint stress (e.g., Weinstock et al., '88) are themselves associated with developmental toxicity, therefore, these represent potential confounders in most previous experiments on ultrasound. Most ultrasound studies have included anesthesia-exposed controls. If ultrasound interacts additively or synergistically, these studies would have detected such effects. However, if ultrasound interacted subtractively with hypothermia-inducing anesthesthetics, such that the hypothermia interrupted ultrasound-induced hyperthermia, these experiments could have missed such effects. No experiments are currently available that can rule out the latter possibility.

The purpose of the present experiment was to test the possible teratogenic bioeffects of varying intensities of continuous wave (cw) ultrasound in the absence of maternal anesthesia or restraint. In order to achieve this, the experiment relied upon conditioned immobilization of the subjects during exposure. Extreme examples of conditioned immobilization are known in the context of the conditioned despair paradigm (Porsolt et al., '77a,b), learned helplessness (Seligman and Beagley, '75; Seligman et al., '75), and reflex immobility (see Klemm, '71). Relying on the principles of inducing these forms of immobility, the present investigation used a milder form of immobility conditioning.

MATERIALS AND METHODS

Nulliparous female Sprague-Dawley CD (VAF) rats (Charles River, Portage, MI) served as subjects. Rats were maintained in a vivarium fully accredited by the American Association for the Accreditation of Laboratory Animal Care at 21 ± 1°C, 50 ± 10% humidity, on a 14/10-hr light/dark cycle on Purina 5001 rat chow and tap water ad lib. Females were acclimated to the laboratory for not less than 2 weeks prior to breeding. Immobility training was conducted prior to placing females with males. Using a water-filled chamber approximately the same size as that of the confinement chamber in the exposure vessel, each female received two days of 15 min and 2 days of 10 min confinement in the training tank. Repetitive confinement induces conditioned immobility, i.e., a cessation of efforts to escape. The resulting behavior is best described as a mixture of floating and propping. Because the chamber is small, rats can partially prop themselves by bracing their legs against opposing walls. However, they cannot elevate themselves above the water because the polished acrylic sides provide only sufficient traction that, when combined with their buoyancy, allows them to remain at the surface without needing to tread water. However, this response is not absolute therefore dams were observed during exposure for movement, body orientation, and number of 180° turns. The day following the last training day females were housed with males. Females were bred with proven sires of the same strain and supplier. Discovery of a vaginal plug was considered embryonic (E) day 0.

On E0, dams were assigned to one of four treatment groups on a weight-matched basis by an experimenter not conducting exposures and encoded on the exposimetry computer so that experimenters were blind to treatment group assignment. Eleven dams were assigned to each group, except the highest exposure group, which had 12 dams.

On E0, dams were placed in individual cages, and their food and water consumption was measured daily throughout gestation. On E3, each dam received refresher immobility training (15 min) in the exposure vessel. After removal from the vessel,

each dam had its abdominal hair depilated using Nair, having had most of their hair removed using electric surgical clippers previously. Depilation was repeated as needed.

Exposure system

The ultrasound exposimetry system was developed specifically for these experiments. The system is described in detail elsewhere (Smith et al., '90). Briefly, a partially focused (measured focal length of 28.9 cm and 3 dB axial depth of focus of 11.2 cm), 4.6 cm diameter, 3 MHz, PZT-4 crystal was mounted in a movable transducer assembly platform approximately 30 cm below the water surface with the ultrasound beam directed upward towards the floating rat. The ultrasound field was calibrated under free-field conditions with a calibrated (at NPL) membrane hydrophone (Marconi model Y-33-7611). For a stationary beam, at the focus that would be at a location within the floating rat, the free-field 90% and 50% intensity beam widths were 1.6 cm and 3.2 cm, respectively. Also, under stationary beam conditions, at the focus, the four continuous wave values of the spatial peak, temporal average intensity (I_{SPTA}) used in this study were 0 (control), 0.1, 2, and 30 W/cm^2, as calculated from the measured instantaneous pressures.

To provide uniform ultrasound exposure to the floating rat's abdominal surface, a modified approach to that used by O'Brien et al. ('82) was used. The rats were trained to remain relatively immobile and placed in an inner confinement chamber measuring 11 × 16 cm constructed of black acrylic. The rat's surface area was estimated to be approximately 7 (width at the widest point) × 8 cm (xiphoid process to the prepuce) on E17 (width varying from 6 to 8 cm from E4-19). A raster scan pattern of the moveable transducer assembly was set at 9 × 15 cm or approximately 1 cm inside each wall, as shown in Figure 1, to ensure that the entire confinement chamber was insonated.

Figure 1 shows, from a top view of the confinement chamber, the pattern of a single raster scan sequence which consisted of 16 9-cm rasters separated by 1 cm. The transducer assembly platform was positioned at the center of the chamber when the rat was placed in it with the ultrasound off. The platform was actuated by two lead screws connected to stepping motors under computer control. At the initiation of the

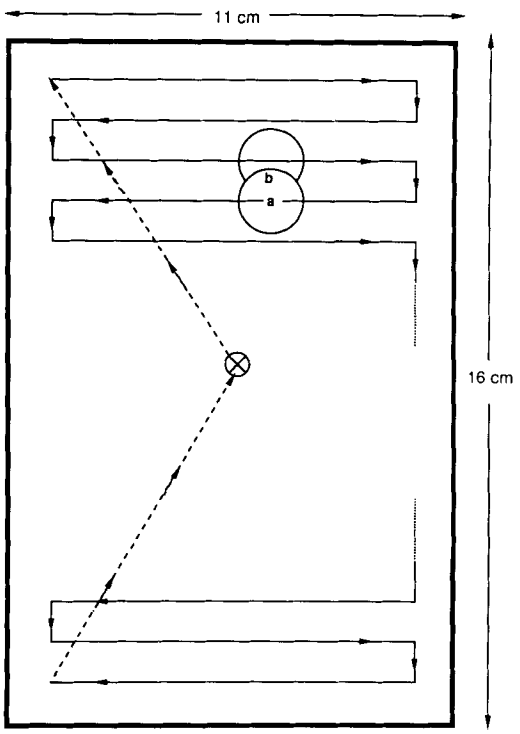

Fig. 1. Top view of the 11 × 16 cm confinement chamber showing the 16 9-cm rasters. The encircled symbol, ×, denotes the starting position. (—›) Ultrasound "off" condition; (—), ultrasound "on" condition. The two 90% beam widths demonstrate the exposure time for the two separate fixed positions. Position 'a' is exposed once to the raster beam, whereas position 'b' is exposed twice.

exposure protocol, the transducer assembly was moved to the upper left corner, which took 116 sec. Upon reaching the corner position, the ultrasound was activated (at I_{SPTA} values of 0, 0.1, 2.0, or 30.0 W/cm^2) and the beam rastered at a rate of 0.2 cm/sec, stepped down 1 cm at a rate of 0.08 cm/sec to the next raster line and rastered across. This was continued for a total of 16 raster lines until the transducer reached the lower left corner, at which time the ultrasound was turned off and the transducer assembly returned to its starting position.

Exposure time can be estimated by evaluating, from the raster scan pattern, the time that a fixed point on an immobile rat's abdominal surface received ultrasound. Assume that the fixed point is on one of the raster lines (point a in Fig. 1) and that the beam width on the rat's abdominal surface

is 1.6 cm, that is, the 90% intensity beam width. The time that the fixed point is within the 90% intensity beam width is determined from a single raster scan to be (1.6 cm)/(0.2 cm/sec) = 8.0 sec. For this case, if the fixed point is midway between two raster scans (point b in Fig. 1), it is exposed twice, thus yielding a total exposure time of 12.0 sec. For the 50% intensity beam width, the total exposure time ranges between 41.0 and 46.5 sec.

Each rat received one raster scan pattern per day on E4-19 for a total of 16 insonation exposures, each lasting 15 min, 7 sec with 1 min, 56 sec transducer positioning time at each end of the scanning pattern for a total of 17 min per session.[1]

Fresh deionized water was placed in the exposimetry vessel daily after testing and allowed to degas overnight. Water temperature was controlled by a Yellow Springs Instruments Proportional Temperature Controller (Model 72) with a YSI model 402 probe set at 35°C and independently checked daily for accuracy with a glass thermometer mounted inside the vessel. Dams were weighed on E0 and E4-20.

Water temperature of 35°C was chosen based on pilot data. In brief, rectal temperatures in six immobilization-trained female rats placed in 37°C water for three consecutive days showed temperature increases averaging 1.1°C 10–15 min after immersion. However, when females were tested in 35°C water for 3 consecutive days rectal temperatures increased an average of only 0.2°C. Therefore, the latter temperature was deemed more suitable.

Teratological procedures

On E20, all dams were euthanized by CO_2 overdose and a laparotomy performed. The uterine horns were exteriorized and the contents examined for implantation sites. Each fetus' position was noted; then it was 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 (1965). 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

Frequency data, such as percentage of malformed offspring and mortality, were analyzed by Fisher's test for uncorrelated proportions (two-tailed). Data such as body weight were analyzed using fixed-effect factorial analyses of variance (general linear model), with litter as the unit of analysis. Split-plot analyses of variance were employed for maternal body weight during gestation and number of 180° turns during exposure with day of observation as the repeated measures factor. In these analyses orthogonal components of the variance–covariance matrix were tested for sphericity as an index of compound symmetry. Where the sphericity condition was not met, F-tests were corrected using the method of Greenhouse–Geisser. For all ANOVAs, a posteriori group comparisons were conducted using Duncan's multiple range test. For malformation analyses conducted on the proportion affected per litter the data were first transformed because of the high frequency of zero values. The procedure of Kirk ('68) was followed to arrive at a transformation. Accordingly, the transformation selected was as follows: $x' = 2 \arcsin \sqrt{x}$ for $x \neq 0$ and $x' = 2 \arcsin \sqrt{1/2n}$, for $x = 0$, where n = number of observations on which the proportion is based.

RESULTS

An analysis of variance (ANOVA) with repeated measures for day performed on maternal body weight during gestation revealed no treatment group effects and no treatment group-related interaction. Similar analyses performed on daily maternal food and water consumption data also revealed no treatment group effects or treatment-related interactions.

The reproductive and fetal outcome data are summarized in Table 1. Insonation exposures were begun prior to implantation, but no evidence of increased pre-implantation loss was found. A non-dose-dependent increase in resorptions was obtained in the lowest exposure group (0.1 W/cm^2) compared with controls. This in turn resulted in

[1]The transducer moves for 19 min (2 min from the origin in the center to the start position located in one corner, 15 min of scanning, and 2 min from the end of the raster pattern back to the origin), but the animal is removed from the chamber as soon as scanning ends. Hence, the last 2 min are not counted as part of the test session.

TABLE 1. Effects of cw ultrasound 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	0.1	2.0	30.0
No. of sperm + females	11	11	11	12
No. of nonparturient dams[1]	1	0	0	2
No. of implants/dam	13.4	12.3	15.1	14.6
No. of resorptions/tot. implants	3/134	16/135	9/166	6/146
% resorbed	2.2	11.9**	5.4	4.1
No. live fetuses	131	119	157	140
No. live fetuses/dam	13.1	10.8	14.3	14.0
Malformations (aggregate)				
No. (%) Total	7/131 (5.3)	1/119 (0.8)**	0/157 (0)**	0/140 (0)**
No. (%) Skel.	3/65 (4.6)	1/56 (1.8)	0/76 (0)	0/67 (0)
No. (%) Visc.	4/66 (6.1)	0/63 (0)*	0/81 (0)*	0/73 (0)*
Malformations (by litter)[2]				
Total	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.01	0.4 ± 0.01
Skeletal	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.01	0.6 ± 0.01
Visceral	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.01	0.5 ± 0.01
Fetal body wt. by litter (g)[2]	3.25 ± 0.06	3.53 ± 0.10*	3.45 ± 0.06*	3.41 ± 0.04

[1]All nonparturient dams were negative for implantation sites.
[2]Values represent the group mean ±SEM.
*Significantly different from controls ($P < 0.05$).
**Significantly different from controls ($P < 0.01$).

a slight reduction in the mean number of live fetuses per dam in this group, but no significant group differences on this measure were obtained.

Malformations were expressed both in toto, i.e., the aggregate number irrespective of litter, and as the mean proportion affected per litter. Considering the aggregate analysis first, Fisher's tests showed significant reductions in malformations in all treatment groups compared with controls. When separated into skeletal and visceral defects, it is evident that this was due to the fact that the insonated groups had no visceral defects, whereas the controls had a low incidence. The visceral defects seen in the controls were: three fetuses with hydronephrosis and one with multiple defects primarily of the neural tube; skeletal: one fetus with digit defects, one with rib defects, and one with kinked tail. The one abnormal insonated fetus was in the 0.1-W/cm^2 group and it had multiple skeletal defects mostly involving missing vertebrae. When the malformation data were analyzed by litter using an ANOVA, no significant treatment group differences in total, skeletal, or visceral malformations were found.

Finally, an ANOVA on fetal body weight revealed a significant group effect ($P < 0.05$). A posteriori group comparisons revealed that this effect was attributable to the low (0.1-W/cm^2) and middle (2.0-W/cm^2) exposure level groups being heavier than controls (both comparisons significant at $P < 0.05$).

During exposure dams were observed for their degree of immobility. A range of behaviors was exhibited. Some dams propped themselves against the sides and remained immobile, except for occasional turning of their heads. Others did not remain entirely immobile. When movement occurred among the latter animals the most common maneuver was to turn 180° in order to face the opposite end of the confinement vessel. These movements were brief and represented only a small fraction of the total exposure time. An ANOVA on 180° turns showed no treatment group effects. The mean (±SEM) number of turns per group per day were: controls = 6.1 (± 1.6), 0.1 W/cm^2 = 8.6 (± 2.5), 2.0 W/cm^2 = 3.2 (± 1.0), and 30 W/cm^2 = 6.8 (± 2.3). The observational data also revealed that the dams' typical posture while immobile was to curve their backs such that their lower extremities were oriented downward in a C-like shape.

DISCUSSION

Although a substantial number of experimental investigations have been conducted on the potential teratogenic and body weight effects of prenatal ultrasound exposure, virtually every study has relied upon anesthetization or physical restraint in order to achieve immobility of the subjects

during exposure. While such procedures succeed in making the subject stationary they do so at the expense of introducing another variable into the study design whose embryonic effects are themselves problematic. One such effect is anesthesia-induced hypothermia. While not all anesthetics cause body temperature reductions, the barbiturates and halogenated inhalant anesthetics do (Gilman et al., '90). In the experiments cited in the Introduction, by far the most widely used anesthetic agent in ultrasound teratologic experiments has been pentobarbital. A distant second is methoxyflurane. Isolated experiments have used ether or a fentanyl–doperiodol mixture. If ultrasound were teratogenic via increased temperature, experiments using temperature-lowering anesthetics could inadvertently attenuate these effects. In an attempt to circumvent this problem, we developed a conditioned immobility procedure in order to expose gravid rats to ultrasound without the use of anesthesia or forced restraint. The data from this experiment showed that such insonation produced no adverse effects on maternal body weight, no increase in pre-implantation embryonic loss, no increase in fetal malformations, and no decrease in fetal body weight. Furthermore, no dose-related increase in resorptions was found. The isolated increase in resorptions noted in the lowest exposure group (0.1 W/cm^2) cannot credibly be regarded as treatment related. Therefore, to the extent that the present experiment accomplished its goal of eliminating anesthesia or forced restraint as factors, it succeeded in showing no grossly embryotoxic effects of intrauterine exposure to incident continuous wave ultrasound at daily exposure levels of up to 30 W/cm^2 I_{SPTA} for 15 min/day on days E4–19 in rats.

During the course of the experiment it became evident that the conditioned immobility procedure, while successful for most rats, did not result in complete immobilization for all rats. When rats turn from facing one end to the other through an angle of 180° during a scan, the effect of this action is to reduce the exposure for some embryos and increase it for others. For example, if a rat turned only once when the raster was exactly halfway through its scan pattern, half the maternal abdomen would receive no exposure, and the other half would receive double exposure. If ultrasound induced embryotoxicity, the double-exposed embryos should be twice as affected and the underexposed embryos should be unaffected. Fetal examination under these circumstances would be expected to produce large within-litter variability ranging from embryotoxicity to no effect. This did not occur, as there was no evidence of any teratogenicity. This suggests that even when maternal movements occur and result in heightened exposure, ultrasound is still not embryotoxic.

In addition, awake rats do not float entirely flat, but rather tend to bend their hindquarters downward. When a rat is positioned with the ventral surface parallel to the waterline, the typical transmission distance between the skin surface and embryos/fetuses is 1–3 cm, whereas when the rat's ventral surface is positioned more perpendicular to the waterline, typical transmission distances are increased to 3–6 cm. The quoted intensities (I_{SPTA}) are free-field, that is, they are the incident intensity values. Assuming total transmission into the rat and an appropriate tissue attenuation coefficient (0.5 dB/cm-MHz), embryos/fetuses of a horizontally positioned dam would be expected to receive insonation 30–65% less than the incident intensity value. Whereas, using the same assumptions, the exposure for the C-positioned rats would be 65–87% less than the incident intensity. Thus, the C-posture would be expected to reduce the actual exposure compared to that found in rats oriented horizontally. However, given the high exposure used here (30 W/cm^2 incident exposure and estimated embryonic exposure, with allowance for tissue and orientation-induced attenuation, of 10.5–21 W/cm^2 for horizontally oriented rats and 3.9–10.5 W/cm^2 for C-positioned rats) it is evident that the actual exposure levels exceed those encountered in clinical obstetrics (Siddiqi et al., '91).

Taken together with other experiments, the present data support the view that even high levels of cw insonation do not induce gross embryotoxicity in rats. Whether effects on CNS development are similarly unaffected by ultrasound must await the results of further investigation into possible developmental neurotoxicity. Such studies are in progress.

ACKNOWLEDGMENTS

We wish to express our sincere appreciation to M.S. Moran and C.A. Blanton for

their expert assistance in the conduct of this experiment. This research was supported by U.S. Public Health Service grants HD21806 and ES07051 from the National Institutes of Health.

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