BIOLOGY OF REPRODUCTION 45, 432–439 (1991)

Effects of In Utero Ultrasound Exposure on the Development of the Fetal Mouse Testis[1]

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ABSTRACT

The effect on the developing fetal testis of in utero exposure to 1-MHz, continuous-wave ultrasound in the spatial peak, temporal average intensity range 0.5–10 W/cm^2 for durations of 400–30 sec on Day 9, 12, or 15 of gestation was determined. Results show that two subtle, yet potentially deleterious, effects occurred: a reduction in the Sertoli cell population and an apparent delay in the cessation of gonocyte mitosis. An increase was also seen in the number of fetal resorptions and stillborn pups per number of implantation sites in the exposed specimens as compared to the sham and cage controls. Because the reduction in testis weight was proportional to decreased body weight and because there was no difference in Sertoli cell numbers due to day of treatment, the testicular effects may reflect a generalized delay in growth. Whether this effect of ultrasound on fetal testis will be translated into an equal reduction in germ cell numbers in the adult testis remains to be determined.

INTRODUCTION

Ultrasound has been extensively employed clinically to monitor the health and well-being of the developing fetus for nearly three decades [1]. It has been estimated that 80% of women in Europe and the United States, for example, undergo at least one sonogram procedure during pregnancy, implying that more than one-half the population receives ultrasound exposure prior to birth [2, 3]. Such a large number of fetal exposures has raised concern for safety and has encouraged several animal studies to investigate possible effects from these exposures [4]. While it is comforting to know that obvious deleterious effects have not been detected from the clinical use of ultrasound, there still remains the possibility that subtle or delayed effects that have not yet been observed may occur. Recently obtained experimental data suggest that potential subtle effects may be produced; insonation of near-term fetal lambs induced changes in the auditory brain stem response [5]. Further, Tarantal and Hendrickx [6, 7] observed changes in white blood cell counts, behavior, Apgar scores, and body weight in neonatal cynomolgus macaques following in utero ultrasound scans carried out at human clinical conditions. Such findings suggest that further investigations are necessary to assess the full import of clinical exposures upon the fetus and to establish guidelines for safe and efficacious employment of this modality for diagnostic purposes.

One mammalian system thus far overlooked in studies of fetal bioeffects of ultrasound is the fetal reproductive system, in particular the fetal gonad. The fetal gonad is known to be highly sensitive to several types of insult to which it may be exposed during development, such as x-radiation [8], diethylstilbesterol (DES) [9–11], ethinyl estradiol [12, 13], and stress [14–16]. These exposures have produced delayed effects that are expressed primarily in the adult animal, as well as abnormalities in reproductive behavior, gonadotropin production, testicular and ovarian morphology, and fertility. The fact that subtle in utero effects on the gonad may go undetected until adulthood, when these abnormalities can be measured and reproductive capacity can be tested, led us to investigate the potential effects of ultrasound on the fetal mouse testis.

MATERIALS AND METHODS

Mating and Animal Care

All animals were housed in the same animal-care room. They were maintained on a 14L:10D cycle with food (Purina rat chow, Ralston-Purina, St. Louis, MO) and water dispensed ad libitum. Males were housed in close proximity to females to insure regular 4–5-day estrous cycles [17].

Female nulliparous ICR:HD (Harlan Sprague-Dawley, Indianapolis, IN) mice 70–100 days of age in proestrus or estrus, as determined by vaginal smears, were mated to proven males in the afternoon. The following morning (Day 0), the males were removed, and females with seminal plugs or sperm-positive vaginal smears were set aside for exposure to ultrasound on Day 9, 12, or 15 of gestation.

Ultrasound Calibration

Figure 1 illustrates the exposure system used, which includes a Hewlett Packard (Palo Alto, CA) signal generator, an AR power amplifier, and a 1-inch-diameter PZT-4, 1-MHz unfocused ultrasound transducer. The transducer acoustic field has a 95% power beam width of 1 cm and a

Accepted April 23, 1991.

Received December 27, 1990.

[1]The authors gratefully acknowledge support of this work by grants from the National Institutes of Health.

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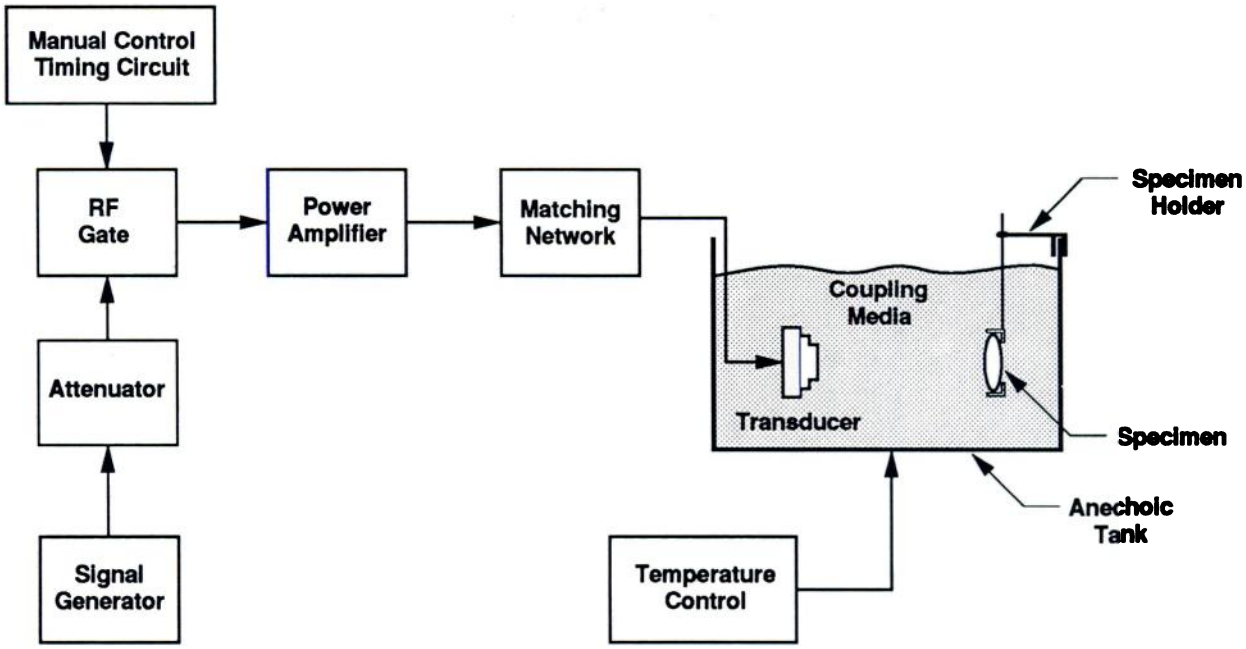


FIG. 1. Schematic representation of the ultrasound exposure system.

3-dB beam width of 1.9 cm. Primary calibration of the acoustic intensity output is carried out by determining the force exerted on a small (1/16-inch diameter) steel sphere and is accurate to ±3% [18]. The calibration is carried out at the same location in the sound field at which the specimen is positioned for the exposure procedure. The ultrasonic beam pattern is determined by the transient thermoelectric method [19].

Experimental Procedures

The dams were anesthetized on the day of exposure with methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ) inhalation; shaved and depilated on the abdomen, back, and sides; and swabbed with a mild detergent solution to effect wetting of the acoustic coupling medium. Depilation is necessary to remove hair stubble, which can trap air bubbles, which would significantly distort the sound field. Each animal, mounted spread-eagle in a specially designed holder, was placed in the Plexiglas exposure tank, which was filled with degassed mammalian Ringer's solution maintained at 37°C, such that the abdomen faced the transducer and was perpendicular to the ultrasound beam axis. The muzzle of the animal protruded above the coupling medium and was fitted into the nose cone of the anesthesia circulator mounted on the holder, which maintained anesthesia throughout the exposure procedure.

A 2 × 3 exposure matrix over the abdominal area was employed to assure uniform exposure over each uterine horn. This was accomplished by maintaining the holder and animal stationary, while moving the transducer to each of the six predetermined positions. A digital position-indicating device provided for maintenance of the 1-cm center-to-center distance between exposure sites.

Groups of six dams, four exposed and two shams, were used for each of the six ultrasound doses, i.e., for each pair of spatial peak temporal average acoustic intensity (I_{SPTA}) and duration of exposure parameters, for each of the 3 days of gestation during which the exposures were carried out. Sham-exposed animals were treated identically to the ultrasound-exposed animals, with the exception that the transducer was not energized. A group of 10 cage control litters (animals neither anesthetized nor restrained) were also assessed to determine if any aspect of the preparation procedure affected the experimental subjects.

Six ultrasound dosages were used: 10 W/cm^2 at 30 sec, 2.5 W/cm^2 at 120 sec, 2.5 W/cm^2 at 180 sec, 1 W/cm^2 at 200 sec, 0.5 W/cm^2 at 400 sec, and 5 W/cm^2 at 90 sec. The 5- and 10-W/cm^2 intensities were chosen as "high" doses to insure occurrence of an effect, the 2.5- and 1-W/cm^2 doses were in the range of therapeutic ultrasound intensities, and the 0.5-W/cm^2 intensity was at the upper range of clinical diagnostic devices. Treatments were given on Day 9, 12, or 15 of gestation. These days of gestation were chosen for exposure because of specific events occurring in gonadal development at those times. On Day 9 of gestation, the primordial germ cells (PGCs) begin migrating from the yolk sac endoderm, where they originate, to the genital ridges, which ultimately develop into the gonads [20]. The PGCs undergo mitosis during and shortly after this migration. The very early stages of sexual differentiation begin on Day 12, when the PGCs move to the peripheral region of the gonadal ridge if the fetus is to be female, or to the central

area if the fetus is to be male [20]. Also, the Sertoli cell precursors are histologically identifiable on Day 12 [21]. On Day 15, the sex cords of the male fetuses, which differentiate into the seminiferous tubules, have become developed and contain the Sertoli cell and the gonocyte precursor cells, which are again dividing mitotically [20].

After the ultrasonic exposure, the dams were removed from the exposure tank, dried, and allowed to recover from the anesthetic. They were then placed three to a cage and returned to the animal quarters until Day 18 of gestation, when they were killed and the fetuses were removed by laparotomy. The following data were recorded: position of fetuses within the uterus, fetal weight, testis weight, number of live fetuses, number of males and females, number of resorptions and stillborns, and presence of any fetal macroscopic abnormalities or deformities.

A detailed study of temperature increases produced in the fetus in response to ultrasound exposure under the same conditions employed in this study has been reported [22].

Histology and Morphometric Analysis

The pairs of fetal gonads were removed, weighed, and placed in Bouin's fixative, then subjected to standard dehydration and paraffin embedding. The specimens were sectioned into 6-μm serial sections, and every fifth section (24 μm between sections) was mounted on a glass slide and stained with hematoxylin and eosin.

Section thicknesses were checked by a direct microscopic method [23]. Ten histologic slides were selected randomly, and section thickness was determined in 3 regions of 2 sections per slide by use of an Olympus Vanox microscope with a 100× planopochromatic objective (1.4 NA). The mean section thickness was 6.01 ± 0.28 μm (SD). Shrinkage due to fixation and embedding was not determined; however, this factor was unlikely to influence the data since pathological conditions such as edema, atrophy, or fibrosis were not present.

After microscopic evaluation of testicles from all males in each litter, two animals from each litter/dose/day were chosen for morphometric analysis, one from each uterine horn. This was performed such that the observer was unaware of the treatment conditions of the specimen. Morphometric analysis of the chosen specimens was by means of JAVA (Jandel Scientific, Corte Madera, CA), a computerized video analysis system that permits the simultaneous viewing of histologic sections and a cursor used for morphometric measurements. Analysis was completed for 30 circular cross sections of seminiferous tubules per testis, 6 from each of the 5 largest consecutive central sections of the testis on each slide. The following data were collected: number of clearly identified Sertoli cell nuclei and gonocyte nuclei per tubule cross section, number of mitotic Sertoli cells, number of mitotic gonocytes, number of degenerating gonocytes, number of multinucleated gonocytes, tubule diameter and area, and percentage of the testis cross-sectional area occupied by the seminiferous tubules (relative tubule area). Total number of cells counted per testis ranged from 28 to 32 gonocytes per section and from 142 to 163 Sertoli cells per section. The relative tubule area ranged from 68% to 82% and showed no statistically significant difference among treatment groups. Data were also recorded for the number of testes exhibiting abnormalities in development and the number of testes with hemorrhages or hypervascularity.

Sertoli cells, gonocytes, and mitotic figures for each were identified according to the criteria of Clermont and Perey [24]. On Day 18 of gestation, gonocytes appear as very large cells located predominantly within the lumen, whereas supporting cells (Sertoli) are found along the basement membrane. The Sertoli cell nucleus was counted rather than the nucleolus because with Bouin's fixative the nucleolus was often confused with large clumps of chromatin. The mean number of Sertoli cell nuclei counted per testis was 744. In fetal testis sections, the Sertoli cell nucleus is often near-round to oval (see Fig. 5), in contrast to the highly irregularly shaped nucleus in adult testis [25]. Mean diameters of Sertoli cell nuclei were determined by measuring two perpendicular diameters per nucleus in 300 clearly visible Sertoli cell nuclei per treatment group. The overall mean diameter was 6.49 μm. No difference in diameter was found between group means ($p < 0.05$). For every gonocyte counted, its nuclear diameter was measured in two perpendicular directions. The mean number of gonocytes counted per testis was 157. The percentage of testis occupied by seminiferous tubules was determined by use of the Jandel Image Analysis System. A defined total area at 66× magnification was circumscribed by the light cursor. Then each seminiferous tubule area within the total defined area was determined and summed.

Degenerating cells were identified by pycnotic nuclei and eosinophilic granulation of their cytoplasm. Multinucleated gonocytes were identified by proximate nuclei surrounded by a large cytoplasmic mass. Hemorrhage was identified by an excessive amount of pooled red blood cells between seminiferous tubules.

The seminiferous tubule volume, the total number of Sertoli cells, and the number of gonocytes per testis were estimated as follows. Following the custom of treating the density of the testis to be sufficiently near to unity (1.04 gm/cm^3, actually) such that testis weight (T_w) can be identified with testis volume [26], the seminiferous tubule volume (V_t) is given by

$$V_t = T_w R \tag{1}$$

where R is the relative tubule area, viz, the area of the testis occupied by seminiferous tubules divided by the total cross-sectional area of the testis section. Considering the tubule to be a circular cylinder of radius r_t and length l_t, we have

$$l_t = V_t/\pi r_t^2 = T_w R/\pi r_t^2 \tag{2}$$

TABLE 1. Percentage of stillborn pups and fetal resorptions per number of implantation sites in sham and ultrasound exposed litters (n) killed on Day 18 of gestation (cage controls = 0.9).

It	n	Day 9	Day 12	Day 15
0	12	1.0	4.8	2.7
200	8	2.2	7.0*	3.8
300	7	20.5*	55.3*	14.7*
450	7	35.0*	38.5*	53.6*

*Treatments are significantly different by analysis of variance, $p \leq 0.05$.

The seminiferous tubule length is then divided by the histosection thickness to obtain the number of unit sections.

The number of Sertoli cells per section was calculated from the method of Abercrombie [27]:

$$\text{true cell count} = \frac{\text{crude count} \times \text{section thickness}}{\text{section thickness} + \text{nuclear diameter}}$$

The probability that differences in the mean nuclear diameters of either cell type for each of the three treatment groups—the exposed, the sham, and the cage controls—was not statistically significant ($p < 0.01$). The total number of Sertoli cells or gonocytes per testis was obtained by multiplying the true cell count by the number of 6-μm unit sections.

Statistical analysis of group differences was by analysis of variance using Statgraphics (Statistical Graphics Corp., Rockville, MD). The various treatments were compared by use of multiple-range analysis of least squares differences, which indicated that the effects analyzed varied significantly with dosage at the 0.05 level.

RESULTS

Ultrasound exposures on Day 9, 12, or 15 of gestation induced dose-dependent effects on general reproductive outcome. There was a significant increase in the percentage of stillborn pups and fetal resorptions in the exposed groups compared with shams for most ultrasound doses (I_{SPTA} in $W/cm^2 \times$ exposure duration in seconds, t), which appears directly related to the energy flux, It (see Table 1). The pregnancy loss was primarily in the form of resorptions with the exception of the 5-W/cm^2 group wherein a large number of stillborn pups occurred.

Although a direct effect on births was evident, no effect was found on total litter size (number of implantation sites), male:female ratio, or number of pups with congenital abnormalities. All three treatment groups had less than 1% incidence of congenital abnormalities.

Table 2 shows the basic effects of the ultrasound parameter I^2t on the fetal testis weight. An inverse relationship is seen between body weight, testicular weight, and the I^2t ultrasound dosage parameter; i.e., increasing ultrasound dosage parameter caused a decrease in testis and body weight. Also found to decrease with increasing I^2t were seminiferous tubule length, volume, and the total number of Sertoli cells per testis.

The incidence of dividing gonocytes also appears to correlate with the energy flux, It. Figure 2 shows that the number of dividing gonocytes per tubule cross section increased significantly at the highest dosage. The data from all three gestational days were pooled by dosage for analysis, as no difference occurred among days of gestation. Sham and cage control values were also pooled. However, total number of gonocytes/testis showed no change across treatments (Table 2). This lack of effect on total number was apparently due to the increase in gonocyte division with increased dosage (Fig. 3).

The gonocyte/Sertoli cell ratio showed a significant increase with increasing I^2t (Fig. 4a) and is described by the linear regression model: gonocyte/Sertoli cell = 2.81×10^{-5} (I^2t) + 0.188. No differences were found among the shams and the cage controls. This increase in cellular ratios is due to an increase in the number of gonocytes per tubule cross section (Fig. 4b, Fig. 5). No difference was found in the number of Sertoli cells per tubular cross section.

The exposed testes also showed an increase in vascular

TABLE 2. Effects of ultrasound exposure (I^2t parameter) on fetal weight, testis weight, seminiferous tubules, and cell populations on Day 18 of gestation.

I^2t	N†	Live pup weight (g)	Testis weight (mg)	N‡	Seminiferous tubule volume (mm^3)	Seminiferous tubule length (mm)	Gonocytes per testis $\times 10^4$*	Sertoli cells per testis $\times 10^5$
Control	56	1.36 ± 0.02^a	1.15 ± 0.08^a	10	0.764 ± 0.02^a	311.8 ± 8.5^a	7.01 ± 0.50	5.39 ± 0.07^a
Sham	206	1.31 ± 0.02^a	1.05 ± 0.04^{ab}	18	0.814 ± 0.01^a	294.0 ± 10.7^b	7.82 ± 0.28	5.76 ± 0.18^{ab}
100	61	1.29 ± 0.03^{ab}	0.93 ± 0.03^{bc}	12	0.698 ± 0.03^b	244.0 ± 24.1^c	7.66 ± 0.53	4.40 ± 0.22^{bcd}
200	75	1.25 ± 0.02^{abc}	0.95 ± 0.04^{bc}	12	0.676 ± 0.03^b	234.3 ± 16.5^{cd}	6.80 ± 0.52	4.75 ± 0.09^{bc}
750	65	1.16 ± 0.07^{cd}	0.90 ± 0.05^{cd}	12	0.674 ± 0.03^b	219.1 ± 16.5^{cde}	6.96 ± 0.54	4.26 ± 0.29^{cd}
1125	57	1.19 ± 0.06^{bc}	0.82 ± 0.03^{cde}	12	0.650 ± 0.02^b	241.5 ± 7.4^c	7.43 ± 0.39	4.19 ± 0.18^{cd}
2250	42	1.04 ± 0.06^{de}	0.75 ± 0.05^{de}	12	0.557 ± 0.03^c	178.4 ± 14.1^e	6.24 ± 0.48	3.60 ± 0.15^d
3000	41	0.98 ± 0.04^e	0.69 ± 0.03^e	6	0.515 ± 0.03^c	184.1 ± 4.1^{de}	6.53 ± 0.23	3.32 ± 0.07^d

$^{a-f}$Means (±SEM) having different superscripts within columns are different ($p < 0.05$).
*No significant difference between means (±SEM).
†Number of fetuses for body and testis weights.
‡Number of testes for morphometry and cell counts.

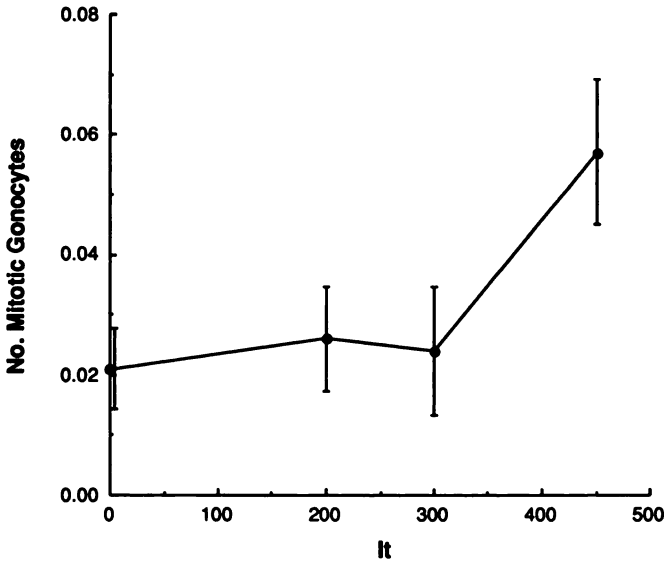


FIG. 2. Number of mitotic gonocytes per round seminiferous tubule cross section vs. the exposure parameter It at Day 18 of gestation.

changes in the form of hypervascularity and occurrence of hemorrhages (Fig. 6), both parenchymatous and per rhexis. These effects were seen in 7% of the cage controls, 13% of the shams, and 21% of the exposed testes; however, no dose dependence on either of the two parameters, It or I^2t, was observed.

DISCUSSION

Extrapolation of the results of this study to clinical exposure of human fetuses requires particular attention to details, since several significant differences exist among the experimental animals and clinical exposures. These include the relationship of the sound-beam diameter to the body and organ sizes; the differences of the pulsed ultrasound

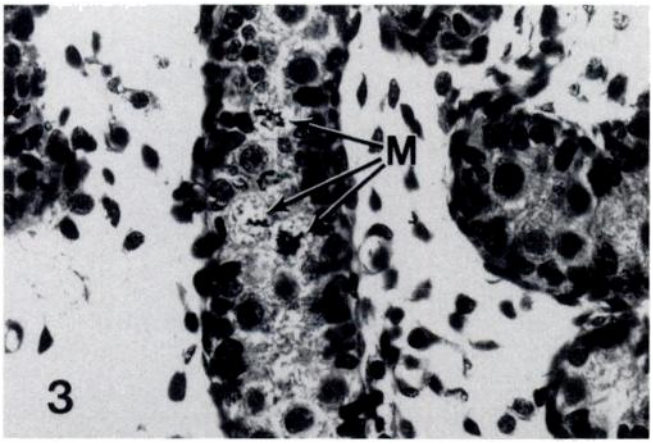
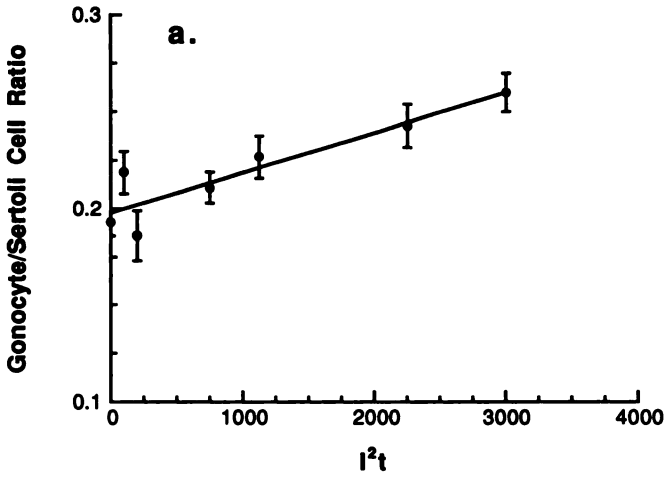


FIG. 3. Seminiferous tubule containing gonocytes in mitotic division (M). Fetus was exposed on Day 12 and killed on Day 18 of gestation. ×400.



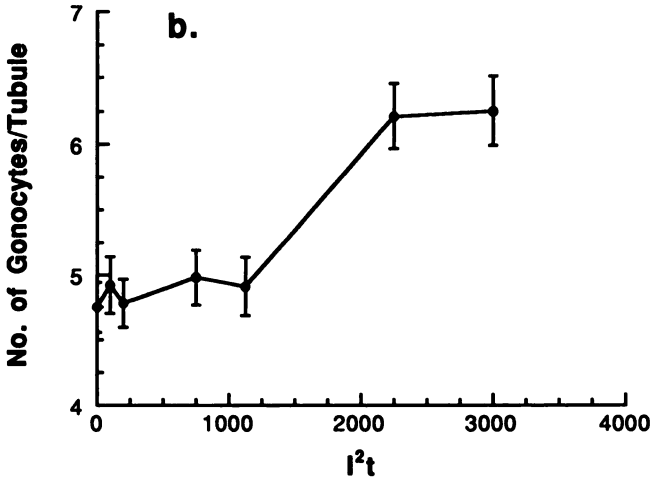


FIG. 4. (a) The gonocyte/Sertoli cell ratio per round seminiferous tubule cross section vs. the exposure parameter I^2t at Day 18 of gestation. (b) The total number of gonocyte nuclei per round seminiferous tubule cross section vs the exposure parameter I^2t.

field employed clinically and the continuous wave fields of these experiments; the differences in the path length the wave travels within the specimen; and the differences due to changes in blood flow and body temperature associated with the anesthesia administered to the experimental animals. However, it should also be noted that the acoustic properties of mammalian tissues have been found to be independent of species such that the physical mechanisms of interaction between the sound waves and the tissues will be the same for mouse and human.

This study has shown that in utero exposure of the mice to ultrasound decreases fetal body weight and testis weight. The animals were exposed to 1 MHz ultrasound at SPTA intensities in the range of 0.5–10 W/cm^2 for corresponding durations in the range 400–30 sec. Testes from treated fe-

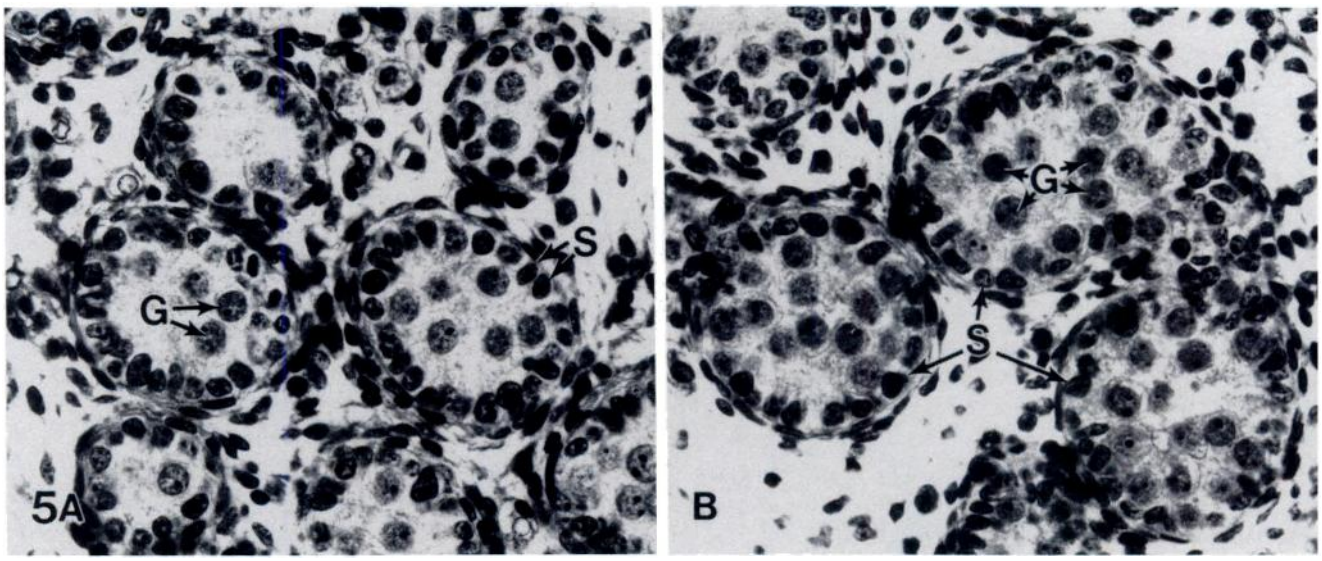


FIG. 5. (A) Round seminiferous tubule cross sections from the testis of a cage control killed on Day 18, showing gonocytes (G) and Sertoli cells (S). ×400. (B) Round seminiferous tubule cross sections from an exposed testis showing Sertoli cells (S) and an increased number of gonocytes (G) compared to those of the cage control. ×400.

tuses on Day 18 of gestation exhibited two subtle, yet potentially deleterious, developmental effects: a reduction in the Sertoli cell population and an apparent delay in the cessation of gonocyte mitosis. As the observed effects depend upon two different dose calculations, It and I^2t, it is postulated that two different physical mechanisms are responsible. Because treatment differences were not found when exposures were made on different days of gestation (Day 9, 12, or 15), it is possible that the testicular effects were due to a general decrease in fetal growth.

The increase in stillborn pups and fetal resorptions was correlated with the energy parameter It, which can be a measure of temperature elevation. Thus it is suggested that the mechanism for this effect is primarily heating resulting from absorption of the sound energy in the tissues. Fetuses exposed on Day 12 of gestation were more sensitive than those exposed on Day 9 or 15.

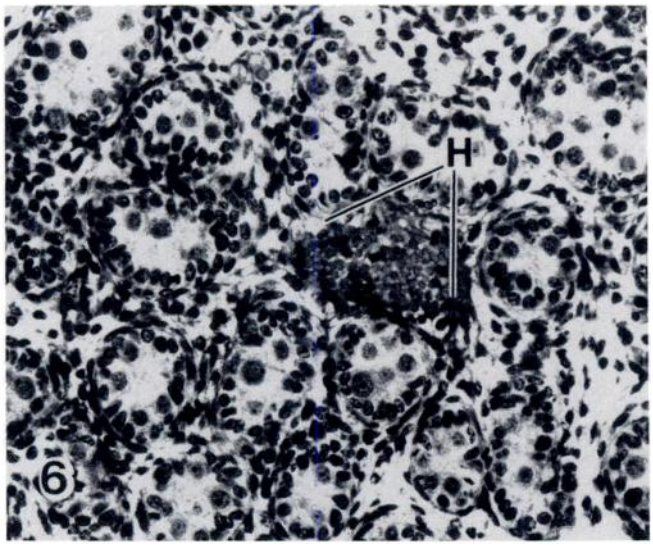


FIG. 6. Area of hemorrhage in a testis of fetus exposed on Day 12 of gestation and killed on Day 18 of gestation. ×290.

The lack of statistically significant differences in occurrence of congenital abnormalities among the cage controls, the shams, and the exposed specimens suggests that although hyperthermia is known to have occurred [22], it was apparently not of sufficient magnitude or duration, or did not occur during the appropriate time of development to induce the incidence of teratologies as reported previously [28–30].

It has been well documented that mitotic division of the gonocyte ceases during Day 16 post coitus (pc) in the mouse and that no further replication takes place until meiosis commences on Day 3 post partum (pp) [20, 31, 32]. It is interesting that the shams and cage controls also contained some dividing gonocytes, an observation in contrast to reports in the literature that gonocyte mitosis stops on Day 16 [20, 32]. The data in Figure 2 clearly show that division had not ceased in the exposed testes. The appearance of the dividing nuclei (Fig. 3) indicates that mitotic rather than meiotic division was taking place. This fact, coupled with an increase in the number of gonocytes, suggests that the effect of the exposure is to delay gonocyte development such that the exposed testes on Day 18 pc have the appearance of Day 16 pc unexposed testes. The consequences of this delay on the prepubertal and adult testis are not clear at this time. As this cellular effect was observed to be solely dependent on the exposure parameter It, it may be a subtle effect that results from the heating of the fetus during the exposure.

The seminiferous tubule of the testis is composed of two

distinct cell types: germ cells and a unique somatic supporting cell type, the Sertoli cell. The Sertoli cell has a number of functions essential to normal germ cell production and fertility. Sertoli cells produce several glycoproteins essential to testicular development and function, such as androgen-binding protein, transferrin, testibumen, inhibin, plasminogen activator, and anti-Mullerian protein [33]. They are also responsible for control of testicular size, form the blood-testis barrier, and, most importantly, support and nurture the developing germ cell [34]. This last function is critical to fertility and daily sperm production as it has been established that Sertoli cells control the number of germ cells produced, and thus a reduction in Sertoli cell number results in a concomitant reduction in the production of germ cells [35]. Furthermore, the number of these cells is established primarily during fetal development, since division ceases in early postnatal life and Sertoli cells are nonproliferating in the prepubertal and adult males of all species [34].

The reduction in testicular size of the ultrasound-exposed testes was primarily a result of reduced length of the seminiferous tubules. The reduction in tubule length correlated with a decreased number of Sertoli cells per testis (Table 2), and both were I^2t dose-dependent. The lower-intensity exposure conditions could possibly be reached in clinical situations, particularly with therapeutic devices.

The exposed testes had a reduced Sertoli cell population on Day 18 of gestation, ranging from 13% to 42% lower than that of the shams. Whether this effect of ultrasound on fetal testis would be translated into an equal reduction in germ cell numbers in the adult testis remains to be determined. Because the reduction in testis weight was proportional to a decrease in body weight, it is possible that ultrasound exposure causes a generalized decrease in growth of the fetus, rather than having a direct effect on the testis. O'Brien [36] previously showed an I^2t dose-dependent fetal weight loss following in utero ultrasound exposure on Day 8 of gestation, over a range of exposure conditions including some similar to those used in this study. The fact that no differences were found in treatment effects due to different days of exposure (Day 9, 12, or 15) also supports the suggestion of a nonspecific effect on growth. However, sometimes testis weight will remain low while body weight recovers following in utero exposure to a toxicant [37]. Germ cells migrate from the gut wall to the gonadal ridge from Day 9 to Day 12 of gestation in the mouse [38]. Sertoli cells are established in the testis between Days 12 and 13, from a population of cells in the glomerulus of the mesonephric tubules [39]. Thus, although an exposure on Day 9 or 12 would not have a direct effect on Sertoli and germ cells within the testis, it could affect the precursor cells located outside the germinal ridge.

The alteration in the gonocyte/Sertoli cell ratio resulted from an increased number of gonocytes per tubule cross section in the experimental animals, while the number of Sertoli cells per tubule cross section remained nearly the same for all treatment groups. Similar results have been produced after in utero exposure to ethinyl estradiol, a common synthetic estrogen that is a component of oral contraceptives [12, 13]. In that case, the change in ratio was due to a decrease in Sertoli cells as well as an increase in gonocytes. The change in ratio of these cell types as shown in our study would most likely result in subsequent degeneration of germ cells, since each Sertoli cell supports a finite number of germ cells [40, 41] and since the Sertoli cells do not divide in the adult testis [42, 43]. Consequently, the Sertoli cells would not be able to compensate for this increase in number of germ cells. That this effect is linearly related to the I^2t parameter suggests a mechanism of action other than heat, possibly hysteresis [44].

The potential long-term consequence of the testicular effects seen in this study (e.g., altered gonocyte/Sertoli cell ratio, increase in the number of gonocytes per tubular cross section, and continuation of gonocyte division) is a decrease in adult daily sperm production, due to the reduced numbers of Sertoli cells to support germ cell development. In the rat, mitosis of Sertoli cells peaks before birth on Day 20 of gestation and ceases on Day 21 postbirth. Thus, the proliferation of Sertoli cells occurs before germ cell differentiation. Because this somatic cell population is established early in testicular development and each Sertoli cell supports a finite number of germ cells [40, 41], the final size of the testis and daily sperm production are limited by these fetal and perinatal events [45, 46]. Orth et al. [35] showed experimentally that a 50% reduction in Sertoli cells per testis in the newborn rat produced a 54% decrease in the spermatid population in the adult testis. Thus, the importance of establishing a proper number of Sertoli cells in the developing testis cannot be overstated.

Except for the incidence of stillbirths and resorptions, no correlation with day of gestation was observed, and it must be assumed, in the absence of further details, that these effects could be caused at any time during development. This is of particular concern as ultrasonic imaging of the fetus is performed at increasingly earlier gestational ages [3]. Although the I^2t doses used in these experiments could be reached using some current clinical devices (Doppler and therapeutic), whether these effects occur after more clinically relevant exposure conditions such as higher frequencies, focused ultrasound fields, or pulsed regimes, has yet to be investigated before risk can be determined for the exposed human fetus.

ACKNOWLEDGMENTS

The authors wish to thank Gerald Bargren, Shu Wen Chen, and Georgia Sebesta for their technical assistance.

REFERENCES

1. NCRP. Biological Effects of Ultrasound: Mechanisms and Clinical Applications. NCRP Report 74, National Council on Radiation Protection and Measurements. 1983; Bethesda: NCRP Publications.

2. Lewin PA, Goldberg BB. Benefits of ultrasound still outweigh risks. Diagnostic Imaging 1989; 11:148–156.
3. Dakins DR. Study leaves FDA in quandry over prenatal ultrasound. Diagnostic Imaging 1990; 12:46.
4. Stewart HD, Stewart HF, Moore RM Jr, Garry J. Compilation of reported biological effects data and ultrasound exposure levels. J Clin Ultrasound 1985; 13:167–186.
5. Siddiqi TA, Meyer RA, Woods TR Jr, Plessinger MB. Ultrasound effects on fetal auditory brain stem responses. Obstet Gynecol 1988; 74:752–756.
6. Tarantal AF, Hendrickx AG. Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque (*Macaca fascicularis*): I. Neonatal/infant observations. Teratology 1989; 39:137–147.
7. Tarantal AF, Hendrickx AG. Evaluation of the bioeffects of prenatal ultrasound exposure in the cynomolgus macaque (*Macaca fascicularis*): II. Growth and behavior during the first year. Teratology 1989; 39:149–162.
8. Mintz B. Embryological phases of mammalian gametogenesis. J Cell Comp Phys 1960; 56(suppl 1):31–48.
9. Bibbo M, Gill WB, Azizi F, Blough R, Fang VS, Rosenfield RL, Schumacher GFB, Sleeper K, Sonek MG, Wied GL. Follow-up study of male and female offspring of DES-exposed mothers. Obstet Gynecol 1977; 49:1–8.
10. Gill WB, Schumacher GFB, Bibbo M. Pathological semen and anatomical abnormalities of the genital tract in human male subjects exposed to diethylstilbestrol *in utero*. J Urol 1977; 117:477–480.
11. McLachlan JA, Newbold RR, Bullock B. Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 1975; 190:991–992.
12. Yasuda Y, Kihara T, Tanimura T. Effect of ethinyl estradiol on the differentiation of mouse fetal testis. Teratology 1985; 32:113–118.
13. Yasuda Y, Konishi H, Matuso T, Tanimura T. Accelerated differentiation in seminiferous tubules of fetal mice prenatally exposed to ethinyl estradiol. Anat Embryo 1986; 174:289–300.
14. Orth JM, Weisz J, Ward OB, Ward IL. Environmental stress alters the developmental pattern of Δ5-3β-hydroxysteroid dehydrogenase activity in Leydig cells of fetal rats: a quantitative cytochemical study. Biol Reprod 1983; 28:625–631.
15. Menendez-Patterson A, Fernandez S, Marin B. *In utero* immobilization stress and its effects on the development, behavior, and sexual maturity of the rat. Revista Espanola De Fisiologia 1975; 38:433–440.
16. Pollard I, Dyer SL. Effect of stress administered during pregnancy on the development of fetal testes and their subsequent function in the adult rat. J Endocrinol 1985; 107:241–245.
17. Whittingham DG, Wood MJ. Reproductive physiology. In: Foster HL, Small JD, Fox JG (eds.), The Mouse in Biomedical Research, vol 3. Normative-Biology, Immunology, and Husbandry. Boston: Academic Press; 1983: 137–164.
18. Dunn F, Averbuch AJ, O'Brien WD Jr. A primary method for the determination of ultrasonic intensity with the elastic sphere radiometer. Acustica 1977; 18:58–61.
19. Fry WJ, Dunn F. Ultrasound: analysis and experimental methods in biological research. In: Nastuk WL (ed.), Physical Techniques in Biological Research, vol 4. New York: Academic Press; 1962: Chapter 6.
20. Rugh R. The Mouse: Its Reproduction and Development. Minneapolis: Burgess Publishing Co; 1968.
21. Dyche WJ. Cytodifferentiation of the fetal mouse Sertoli cell. Anat Rec 1975; 181:349.
22. Carnes KI, Drewniak JL, Dunn F. *In utero* measurement of ultrasonically induced fetal mouse temperature increases. Ultrasound Med Biol 1991 (in press).
23. Weibel ER. Steriological Methods, vol. I. Practical Methods for Biological Morphometry. London: Academic Press; 1979: 139–150.
24. Clermont Y, Perey B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Amer J Anat 1957; 100:241–267.
25. Russell L, Ettlin RA, Sinha Hikim AP, Clegg ED. Histological and Histopathological Evaluation of the Testis. Clearwater: Cache River Press; 1990: 29–37.
26. Wing T, Christensen AK. Morphometric studies on rat seminiferous tubules. Am J Anat 1982; 165:13–25.
27. Abercrombie M. Estimation of nuclear population from microtome sections. Anat Rec 1946; 94:239–247.
28. Pennycruik PR. The effects of acute exposure to high temperatures on prenatal development in the mouse with particular reference to secondary vibrissae. Aust J Biol Sci 1965; 18:97–113.
29. Chernoff GF, Goldern JA, Seymour MA. Neural tube closure in mouse embryos following a hyperthermic exposure. In: Proceedings of the DW Smith Conference; 1985; Santa Fe, NM.
30. Webster WS, Edwards MJ. Hyperthermia and the induction of neural tube defects in mice. Teratology 1984; 29:417–425.
31. Nebel BR, Amarose AP, Hackett EM. Calendar of gametogenic development in the prepubertal male mouse. Science 1961; 134:832–833.
32. Sapsford CS. Changes in the cells of the sex cords and seminiferous tubules during the development of the testis of the rat and mouse. Aust J Zool 1962; 10:178–192.
33. Griswold MD, Morales C, Sylvester SR. Molecular biology of the Sertoli cell. In: Clarke JR (ed.), Oxford Reviews of Reproductive Biology, vol. 10. New York: Oxford University Press; 1988: 120–161.
34. Plöen L, Ritzen EM. Fine structural features of Sertoli cells. In: Van Blerkom J, Motta PM (eds.), Ultrastructure of Reproduction. Boston: Martinus Nijhoff Publishers; 1984: 67–74.
35. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 1988; 122:787–794.
36. O'Brien WD Jr. Dose-dependent effect of ultrasound on fetal weight in mice. J Ultrasound Med 1983; 2:1–8.
37. Anand S, Van Thiel DH. Prenatal and neonatal exposure to cimetidine results in gonadal and sexual dysfunction in adult males. Science 1982; 218:493–394.
38. Guraya SS. Recent progress in the morphology, histochemistry, biochemistry, and physiology of developing and maturing mammalian testis. Int Rev Cytol 1980; 62:187–306.
39. Zamboni L, Upadhyay S. The contribution of the mesonephros to the development of the sheep fetal testis. Am J Anat 1982; 165:339–356.
40. Russell L. Sertoli-germ cell interactions: a review. Gamete Research 1980; 3:179–192.
41. Bardin CW, Cheng CY, Musto NA, Gunsalus GL. The Sertoli cell. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 1. New York: Raven Press; 1988: 941–979.
42. Steinberger A, Steinberger E. The Sertoli cells. In: Johnson AD, Gomes WR, Vandemark NL (eds.), The Testis. New York: Academic Press; 1977: 371–399.
43. Orth JM. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anat Rec 1982; 203:485–492.
44. Johnston RL, Dunn F. Ultrasonic hysteresis in biological media. Radiat Environ Biophys 1981; 19:137–148.
45. Berndtson WE, Igboeli G, Parker WG. Relationship of absolute numbers of Sertoli cells to testicular size and spermatogenesis in young beef bulls. Biol Reprod 1987; 37:241–246.
46. Berndtson WE, Thompson TL. Changing relationships between testis size, Sertoli cell number and spermatogenesis in Sprague-Dawley rats. J Androl 1990; 2:429–435.