

Evaluation of the Bioeffects of Prenatal Ultrasound Exposure in the Cynomolgus Macaque (*Macaca fascicularis*): III. Developmental and Hematologic Studies

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ABSTRACT The multiple applications of diagnostic ultrasound in obstetrics have resulted in a continued rise in the prenatal population exposed each year. Although human epidemiologic and experimental studies with various animal models have not consistently documented any significant, reproducible findings related to clinically relevant exposures, technologic changes in scanning equipment and gaps in our knowledge regarding the interaction(s) of ultrasound with tissues emphasize the need to pursue safety issues. Studies with nonhuman primates have provided information on the potential for pre and postnatal effects on offspring exposed repeatedly during gestation (ATL MK 600, 7.5 MHz, $I_{SPTA} = 27 \text{ mW/cm}^2$; $I_{SPPA} = 85 \text{ W/cm}^2$; Estimated power = 12 mW—scanned for 10 min 5 times weekly gestational day [GD] 20–35; 3 times weekly GD 36–60; once weekly for 20 min GD 60–150). These studies have indicated transient effects on body weight, white blood cell counts (WBCs), and muscle tone postnatally. In an effort to confirm these findings and focus on hematologic changes, a second series of studies was initiated using the same exposure conditions ($N = 22$; 11 exposed, 11 sham controls). Data derived from both studies were combined and confirmed transient reductions in body weights for infants up through 4 months of age ($P \leq 0.03$); no statistically significant differences in muscle tone were noted. Similar to the original findings, WBCs were transiently reduced on days 3 ($P \leq 0.02$) and 21 ($P \leq 0.05$); prenatal sampling indicated a significant difference between the groups on GD 140 ($P \leq 0.04$). No direct effects were evident in bone marrow aspirates collected on postnatal days 3, 9, and 21 ± 1 . Although animals were able to compensate for these observed changes and remained unaffected by their occurrence, additional studies will be required to further our understanding of this phenomenon.

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The applications of diagnostic ultrasound in obstetrics have expanded during the past ten years particularly since the addition of new modalities such as pulsed Doppler and color flow imaging. These new applications have further enhanced the usefulness of this technique as more fetal physiologic information is accessible, particularly in regards to blood flow dynamics and cardiovascular development and function (Jaffe and Warsaw, '92). Although human epidemiologic studies have not shown an association in the incidence of congenital anomalies and the

rise in the use of ultrasound (Brent et al., '91; Ziskin and Petitti, '88), experimental evidence suggests that the interaction of ultrasound with developing biological systems is not fully understood (AIUM Bioeffects Committee Report, '88). Of concern are the subtle and/or long-term manifestations of

Received August 14, 1992; accepted October 5, 1992.

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frequent intrauterine exposure, particularly when considering those areas of the fetus that develop throughout gestation such as the brain and bone (Tarantal and O'Brien, '92). This issue remains pertinent as the percent of the prenatal population exposed each year continues to rise and technologic advances potentiate changing output characteristics of commercial imaging systems. Both of these factors can result in an overall increase in fetal exposure; the acoustic outputs for pulsed and color Doppler are considerably greater than for routine imaging, particularly when combined ("triple mode"). This emphasizes the need to pursue questions related to diagnostic ultrasound and soft tissue interactions in an effort to confirm that unwanted effects do not occur.

Prior studies have been performed with various mammalian species in an effort to identify the interactions that may occur with ultrasound exposure, both in regard to thermal mechanisms (Abraham et al., '89; Bosward et al., '90; Carnes et al., '91; Carstensen et al., '90; Drewniak et al., '89; Lele '75, '79; Tarantal et al., '92) and acoustic cavitation (occurrence of gaseous bubble formation) (Child et al., '90; Hartman et al., '90). Although there is currently little information available on the cavitation phenomenon *in vivo*, a significant amount of data has been generated on the heating that may occur with various imaging techniques (NCRP Report #113, '92). In addition, studies have focused specifically on the potential long-term manifestations of frequent intrauterine exposure with an emphasis on the potential for developmental toxicity (teratogenicity) with both murine and non-human primate models (Brent et al., '91; Stewart et al., '85). Some reports have shown that exposure of gravid rodents to high levels of ultrasound have not yielded any significant changes whereas other studies with exposures at much lower intensity levels (in some cases, diagnostically relevant) have suggested the possibility of an effect. Further, there have been studies where the original findings could not be duplicated, either in other or within the same laboratories (Tarantal and O'Brien, '92). These results emphasize the complexity when examining these issues, part of which is dependent upon the numerous components that must be considered when attempting to define "exposure" or "dose."

These include the physical and biologic properties of the tissues interrogated including the stage of gestation, the output characteristics of the exposure system, the potential attenuation of the beam which is dependent upon the interfaces in its path, and the resultant acoustic energy which reaches the tissue(s) of interest. Documentation of low fetal body weight after *in utero* exposure has, for example, been documented in multiple species including the mouse, rat (Stewart et al., '85), and macaque (Tarantal and Hendrickx, '89 a,b), although this has not been a consistent observation (Child et al., '89; Stewart et al., '85).

Although studies with murine species can address specific questions in regard to tissue interactions, they have a limited application to the human clinical setting primarily due to their size differential. The macaque model has been designed to closely simulate the human exposure, particularly in regard to the methods incorporated for imaging (Tarantal and Hendrickx, '89a,b). Nonhuman primates have been used extensively in biomedical research, and have been established as an excellent model for the human based on their close similarities particularly with respect to developmental and reproductive features (Hendrickx and Binkerd, '90). Prior studies with this species have shown significant, although transient, effects on neonatal monkeys exposed repeatedly throughout gestation including reduced body weights, increased muscle tone, atypical activity patterns, and reductions in white blood cells (WBCs) (Tarantal and Hendrickx, '89a,b). The WBC findings were considered a particularly significant observation which raised questions related to a possible thermal impact on the fetal bone marrow. An effect on the bone marrow was suggested by the fact that (1) both neutrophils and monocytes were reduced since both cell populations are derived from the same immature myeloid progenitor, the colony forming unit-granulocyte-macrophage (CFU-GM), and (2) heat production has been reported to be potentially greatest at soft tissue/bone interfaces (and within the bone itself) (Lehmann et al., '67; NCRP Report #113, '92). This point is particularly relevant when considering the fetus, as changes in ossification over time imply that there may be a greater thermal impact as the developing skeleton ossifies, particu-

TABLE 1. Output characteristics (water values) for the ATL MK 600 Ultrasound System under scanned conditions with a 720A short focus 7.5 MHz scanhead¹

Parameter	
Center frequency	6.0 MHz
Bandwidth	1.6 MHz
PRF	1.0 kHz
PD	344 ns
6 dB in-plane beam width	0.94 cm
6 dB out-of-plane beam width	1.4 cm
p_c	2.3 MPa
p_r	1.4 MPa
PII	27 $\mu\text{J}/\text{cm}^2$
I_{SPTA}	27 mW/cm^2
I_{SPPA}	85 W/cm^2
Estimated source power	12 mW
MI	0.2

¹PRF = pulse repetition frequency; PD = pulse duration; p_c = peak compressional pressure; p_r = peak rarefactional pressure; PII = pulse intensity integral; I = intensity; SPTA = spatial peak temporal average; SPPA = spatial peak pulse average; MI = Mechanical Index.

larly during the third trimester. Additional studies have supported the hypothesis that these effects are confined to the second and/or third trimester, since no hematopoietic changes were noted when ultrasound exposure was confined to the period of organogenesis (Tarantal and Hendrickx, '90). In an effort to confirm these findings and to further pursue the potential mechanism(s) responsible for their occurrence, a second series of studies was proposed which focused specifically on the parameters noted above under the same exposure conditions. The studies described were, therefore, designed to assess (1) whether the effects noted in prior studies could be duplicated, (2) if the WBC changes could be detected in utero, and (3) whether a direct impact on bone marrow could be identified.

MATERIALS AND METHODS

Ultrasound and exosimetry systems

An Advanced Technology Laboratories, Inc., (ATL) MK 600 Ultrasound Imaging System was used for these studies. All scanning was performed with a 720A short focus transducer at 7.5 MHz. The 720A has a focal length of 2.0–2.7 cm, a focal area of 0.82–0.95 cm^2 , and an entrance diameter of 6.4 mm. Acoustical output for the system in scan mode (measured in water, see details below) is described in Table 1.

A semi-automated exosimetry system was designed specifically for these studies in order to monitor the output of the ATL

MK 600, as previously described (Tarantal et al., '92). Briefly, the exosimetry system employs a CompuAdd 316s computer with a Waveform Acquisition and Arbitrary Generator (WAAG II) (Markenrich Corp., Duarte, CA), a PIO24, and custom-built pulse repetition period (PRP) boards installed. All calculations were performed by the CompuAdd 316s computer (Austin, TX) which features a 386sx microprocessor operating at 16 MHz and a 387sx math coprocessor to enhance the speed of floating-point operations and provides 32-bit precision. The WAAG II board is the analog-to-digital converter operating at 40 MHz with 8-bit precision. The PIO24 is a basic inport/output board with a 24 pin "D" connector. The PRP board was specially designed to acquire a trigger signal from an inductor coil located next to the scanhead and calculate the PRP. When measuring output, the transducer was coupled to the membrane side of the water tank with Aquasonic® gel (Parker Laboratories, Inc., Orange, NJ). All measurements were performed in accordance with the procedures in the revised AIUM/NEMA Acoustic Output Measurement Labeling Standard for Diagnostic Ultrasound Equipment (AIUM approved; October 1988) and were performed automatically by the computer after the hydrophone was placed in the desired position. Calibrations included the following parameters: center frequency, bandwidth, pulse repetition frequency, pulse duration, peak compressional and rarefactional pressure, pulse intensity integral, spatial peak temporal average intensity (I_{SPTA}), spatial peak pulse average intensity (I_{SPPA}), and estimated source power (see Table 1). Scanhead replacement which was required periodically during these studies did not appear to alter the output characteristics. Calibrations were performed at multiple timepoints during and at the termination of the studies.

Animals

Normally cycling adult female long-tailed macaques (*Macaca fascicularis*) with a history of prior pregnancy were bred to proven male breeders every other day for two hours over a three-day period during mid-cycle with the last day of mating considered gestational day (GD) 0 (Tarantal and Hendrickx, '89a). Pregnancy was determined by radioimmunoassay of macaque chorionic gonadotropin on GD 18 ± 2 . All procedures

employed within the study conformed to the requirements of the Animal Welfare Act. The California Regional Primate Research Center (CRPRC) is fully accredited by the Association for Accreditation of Laboratory Animal Care; all study protocols are approved prior to implementation by the Institutional Animal Use and Care Committee at the University of California at Davis. Activities related to animal care (diet, housing) were performed as per standard CRPRC operating procedures. Pregnancy in the long-tailed macaque is divided into trimesters by 55 day increments with GD 0–55 representing the first trimester, GD 56–110 representing the second, and GD 111–165 representing the third (term ~GD 165).

A total of 22 animals was incorporated in these studies. Animals exposed to diagnostic ultrasound ($N = 11$) were chair-restrained (Golub and Anderson, '86) and scanned in the same format as described previously (5 exposures weekly from GD 21–35 ± 2 for 10 min, 3 exposures weekly from GD 36–60 ± 2 for 10 min, and one exposure weekly from GD 61–150 ± 2 for 20 min; organogenesis for this species ~GD 20–50) (Tarantal and Hendrickx, '89a,b). Controls ($N = 11$) were chair-restrained and "scanned" with the unit placed on *standby* during the same time intervals. All embryos/fetuses were assessed for viability and normative growth during the exposures which were continuous (Tarantal and Hendrickx, '89a,b). A cesarean section was performed on GD 152 ± 2 and simian Apgar scores assessed at 1, 5, and 10 min of life. A complete physical and morphometric evaluation was performed at birth and all infants were placed in the nonhuman primate nursery for the duration of the study, with body weights obtained daily. Readers are referred to prior publications for details on these procedures (Tarantal and Hendrickx, '89a,b).

Hematology

Fetal blood samples (FBS; 0.3–0.5 ml) were collected by ultrasound-guided cardiocentesis from 12 fetuses (6 exposed, 6 controls) on GD 120 and 140 (Tarantal '90, '92). FBS were placed directly into microtainer tubes with ethylene diaminetetraacetic acid (EDTA) (Bectin, Dickinson, and Co., Rutherford, NJ) and evaluated with a Serono Baker Diagnostic System (Allentown, PA).

Parameters assessed included a WBC count and a differential. Slides were stained with Wright-Giemsa for morphologic evaluation. Blood samples (0.5 ml) were collected from a femoral vein in hand-restrained infants ($N = 19$; 9 exposed and 10 controls) for WBC counts and a differential on days 2 (± 1), 9, 21, 30 (all ± 2), then 1, 2, 3, and 6 months of age. The same methods for evaluating FBS were incorporated.

Infants were immobilized with ketamine hydrochloride (15 mg/kg) on days 3, 10, and 21 ± 1 of life for sterile aspirates of bone marrow (~1.2 ml). Bone marrow was aspirated into sterile heparinized syringes from alternating humeri (i.e., right humerus day 3, left humerus day 10, etc.) using standard CRPRC collection techniques; 1 ml of bone marrow was placed directly in sterile Iscove's modified Dulbecco's media (IMDM) for culture according to established criteria, as previously described (Ash et al., '81; Zanjani et al., '91). The cells were cultured in 0.9% methylcellulose in the presence of 30% fetal calf serum, IMDM, and erythropoietin (1 U/ml), and incubated at 37°C, 5% CO₂ in humidified air in order to determine the number and growth of CFU-GM progenitors by colony formation in methylcellulose. Smears were made for the determination of the relative percentages of immature and mature myeloid elements by direct examination of stained bone marrow (myeloid:erythroid [M:E] ratios). An additional 0.2 ml sample was collected on day 30 ± 1 for M:E ratios. All smears were stained with Wright-Giemsa for morphologic evaluations.

Behavioral testing

Neurobehavioral test

A standardized neurobehavioral test battery (NBT) was performed on days 1–10 as previously described (Tarantal and Hendrickx, '89b). The NBT is designed to evaluate the development and regression of reflexes and simple behavior patterns in addition to muscle tone and motor development. Muscle tone is evaluated both with ("elicited") and without ("observed" or spontaneous) manual intervention and includes observations pertaining to the head, shoulders, hips, knees, elbows, fingers, and toes. The initial examination was performed 5–6 hr post-delivery, then daily from 12–1 pm. These studies were performed in order to confirm results of prior investigations where a statistically significant increase in

observed tone was noted on days 1, 2, and 4 of life (Tarantal and Hendrickx, '89b).

Observation cage

Spontaneous gross motor behavior and activity patterns were observed weekly for 18 weeks in a $34 \times 34 \times 750$ cm observation cage, as previously described (Tarantal and Hendrickx, '89b). Activities evaluated included climbing, locomotion (crawl, walk), rest (lie, sit), and vertical activities (stand, sprawl, cling); cage exploration; social interactions; and vocalizations during 15–30 sec intervals for a total observation time of 7.5 min. Each observation was performed once weekly during 12–1 pm. These studies were included in order to expand the results of prior investigations where an increase in the percent of time exposed animals were found sitting on the bottom of the testing cage (quiet rest) was noted.

All hematology and behavioral evaluations in addition to animal care functions were performed by individuals blinded to the study groups and animal assignments.

Data analyses

Group differences were assessed with repeated measures analyses (analysis of variance [ANOVA]) and the Student's *t*-test, where appropriate. All statistical evaluations were performed on a Macintosh SE or IIsi with a statistical software package (Statview 512+, Brainpower Inc., Calabasas, CA). All differences were considered statistically significant at $P \leq 0.05$.

Data from the current study and those obtained from prior investigations (13 treated, 10 controls) were combined in order to increase the total *N* to 22 exposed and 20 controls for the following parameters only: Apgar scores, body weights, postnatal WBCs, muscle tone (derived from the NBTs), and observation cage activities.

RESULTS

There were no indications of gross malformations either pre or postnatally in any of the exposed or control animals on study. This further supported original findings where a similar outcome was noted (Tarantal and Hendrickx, '89a). There were two abortions in the exposed group, one on GD 105 and one on GD 129. One control infant died at delivery (respiratory complications) and one at two weeks of life (hypothermia).

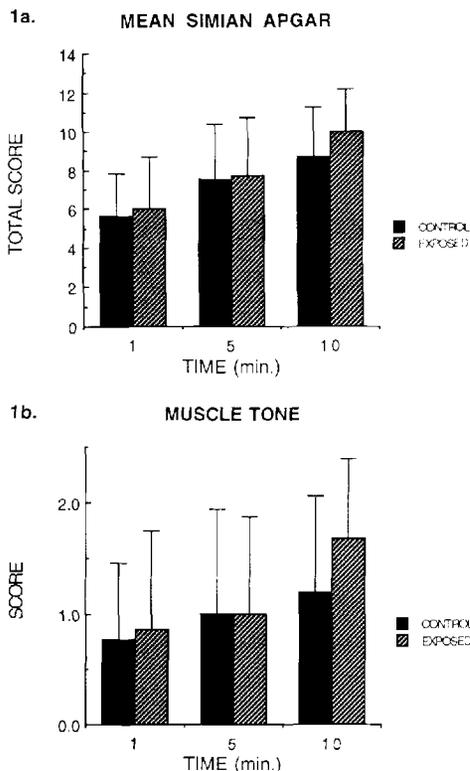


Fig. 1. a: Total scores obtained in the simian Apgar at 1, 5, and 10 min of life for sham control and ultrasound-exposed neonatal monkeys (maximum scores achievable = 12; mean \pm SD shown). b: Scores obtained for muscle tone only at 1, 5, and 10 min of life (maximum score = 2; mean \pm SD shown).

All pre and postnatal deaths were considered unrelated to ultrasound exposure.

Simian Apgar scores

Although prior studies had shown a statistically significant elevation for exposed animals at 10 min for the overall score ($P \leq 0.05$) and that obtained for muscle tone ($P \leq 0.01$), no statistically significant changes were noted with the addition of the second group of study animals. A trend towards increased scores for exposed animals was, however, suggested with a moderate elevation at 10 min for muscle tone (Fig. 1a,b).

Body weight

Significant differences in body weights, both at birth and throughout the first 4 months of life were noted ($P \leq 0.03$), confirming the original findings ($P \leq 0.03$) (see Fig. 2). A similar growth trend was shown

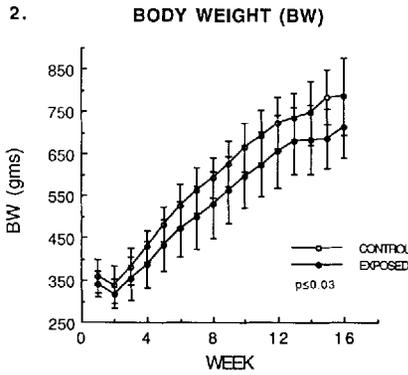


Fig. 2. Weekly body weights (gm) for sham control and ultrasound-exposed infant monkeys from birth to 4 months of age (mean \pm SD). Significant differences were detected during this time ($P \leq 0.03$), with nonsignificant changes noted thereafter.

between dose groups, with the anticipated decline in body weight during the first week of life, which is typical for newborn monkeys.

Hematology

Hematologic differences in regard to neonatal WBCs were similar for combined studies when compared to those reported previously (Fig. 3a,b). There was a statistically significant difference noted on days 3 ($P \leq 0.02$) and 17 ($P \leq 0.05$) (Fig. 3a) when comparing control and exposed animals, with exposed infants showing a reduced mean WBC count. This difference was no longer evident by one month of age, when exposed animals showed a mean WBC count which was greater than concurrent controls. There were no biological or statistically significant differences noted during the later time-points (2–6 mos; Fig. 3b).

Additional sampling during the fetal period (fWBC; GD 120 and 140) revealed a significant reduction in WBC counts for exposed fetuses on GD 140 ($P \leq 0.04$) (Fig. 3c). Although similar numbers were detected on GD 120 for both groups, the differences detected on GD 140 appear to be related to a lack of increase in cell numbers over time (i.e., from GD 120–140). While control fetuses approximately doubled their white cell counts, exposed fetuses showed little change. Examination of individual animals further illustrates this difference (Fig. 3d). The trend for controls was a continued rise on GD 140 (graphed as day -10) with a peak in cell numbers on postnatal day 3.

WBCs were noted to decline thereafter until roughly one month of age. Two of the three exposed animals represented did not show a rise in white cell counts until postnatal day 3 and one until postnatal day 9, with approximate normalization by one month of age.

Results of bone marrow assessments did not reveal any statistically significant differences between dose groups (see Table 2). Colony growth of CFU-GM was roughly comparable for both groups with a marginally smaller number of mean colonies detected for exposed animals on day 3 \pm 1 (90.1 ± 53.5) compared to controls (104.8 ± 67.6). Day 10 \pm 1 (exposed: 98.8 ± 69.2 , control: 105.5 ± 84.8) and day 21 \pm 1 (exposed: 108.1 ± 68.2 , control: 101.7 ± 70.7) were also similar. Morphologic evaluation of stained bone marrow showed the M:E ratios, completeness of the myeloid to erythroid series, and appearance of megakaryocytes and mitotic figures were similar between dose groups. Two exposed infants sampled on day 3 \pm 1 of life were noted with inadequate cellularity by smear analysis; one was identified with the lowest CFU-GM colony growth (day 3: 9.8 ± 1.5 ; day 10: no formed colonies detected; day 21: 14.3 ± 0.3 ; WBC data shown as "Exposed #1" in Fig. 3d) whereas the other infant displayed consistent colony formation (day 3: 89.3 ± 6.7 ; day 10: 97.7 ± 11.1 ; day 21: 83.0 ± 3.0) which was within the mean range anticipated for this age.

Behavioral testing

Neurobehavioral test

Only marginal increases (not statistically significant) in observed muscle tone were noted on day 1 of life when comparing control and exposed neonates (see Fig. 4). These differences were confined to observations on day 1 only, as results from all other days were identical for both elicited and observed tonus.

Observation cage

Although the overall percentage of quiet activities was not significantly different between the dose groups, a breakdown of this category (lie + sit + cling divided by total rest [lie + sit] and vertical activities [cling + stand + sprawl]) supported the original findings which showed an increase in the amount of time the exposed infants were found sitting on the bottom of the testing

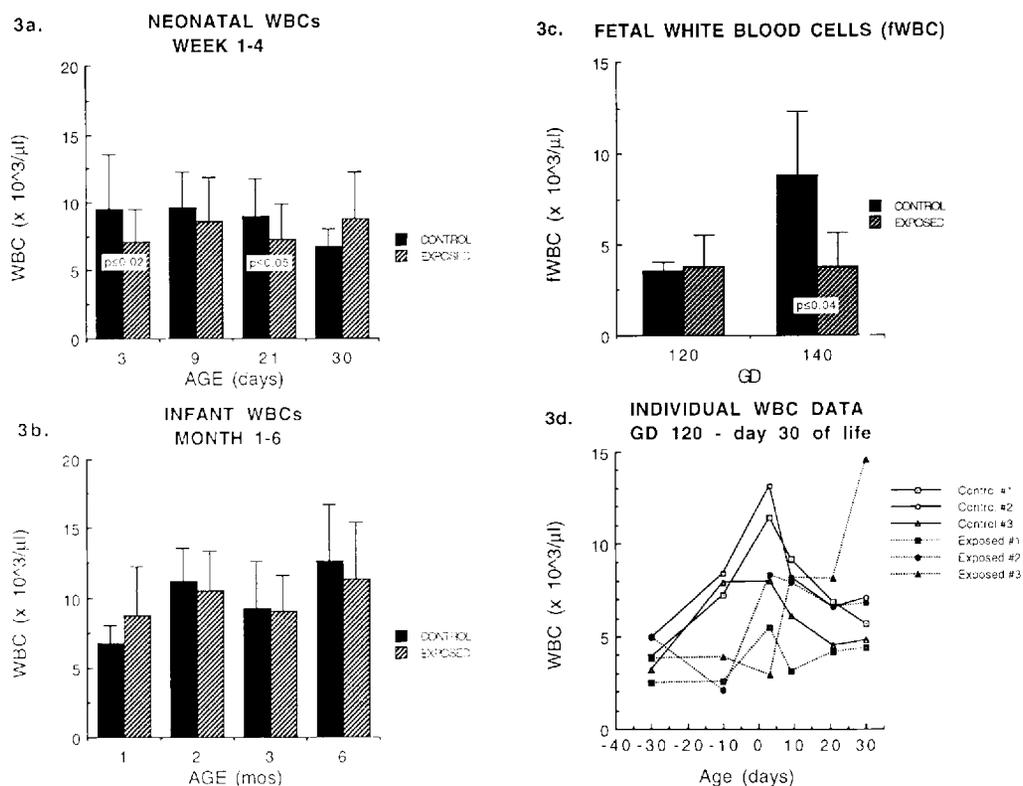


Fig. 3. **a:** Neonatal WBCs during the first months of life. Note statistically significant differences when comparing sham control infants to those exposed to ultrasound prenatally (days 3 and 21 \pm 1). **b:** Infant WBCs from 1–6 months of life. There were no differences detected during this time period between the groups. **c:** Fetal white blood cells (fWBC) from sham control and ultrasound-exposed macaque fetuses collected by ultrasound-guided cardiocentesis on gestational days (GD) 120 and 140 \pm 2 (mean \pm SD). Note statistically significant difference in fWBCs on GD 140 and the lack of increase in cell numbers for exposed fetuses when com-

paring GD 120 to 140. **d:** Individual WBC data for 3 control and 3 exposed animals from GD 120 [–30 days] to 1 month of age. Although GD 120 values were comparable for both study groups, there were significant differences detected on GD 140 [–10 days]. While control fetuses showed a rise in WBC prenatally, no comparable changes were noted in exposed fetuses. White cell counts for most fetuses continued to rise when sampled on postnatal day 3 \pm 1 and all approximately normalized by one month of age. One animal (Exposed #2) showed no appreciable difference in white cell counts until postnatal day 9.

age (Fig. 5a), versus lying (a more immature form of rest activities; comparable in both groups), standing (Fig. 5b), clinging (a more immature form of vertical activity; Fig. 5c), or sprawled on the sides of the test apparatus (Fig. 5d). This was particularly evident when the testing period was extended to 18 weeks, as a greater difference in the mean response between groups was noted during this period (see Fig. 5a). During this stage, when more exposed animals were found quietly sitting on the bottom of the cage, controls were more actively engaged in climbing activities which involved either clinging to or sprawled along the side of the test cage.

DISCUSSION

Developmental hematopoiesis in the human embryo and fetus has been well-described during the course of gestation (Kelemen et al., '79). Initially established in the yolk sac, blood cells are restricted to its wall and the allantoic diverticulum but can be found thereafter in the developing liver and spleen. The current assumption is that hematopoietic stem cells (HSC) gain access to the embryonic circulation via the vitelline and allantoic circulations then migrate to the hepatic anlage, although the seeding phenomenon still remains somewhat controversial.

TABLE 2. Evaluation of bone marrow aspirates obtained from sham control and ultrasound-exposed infant long-tailed macaques (*Macaca fascicularis*) during the first month of life¹

Age (days)	Group (N)	M:E ratio (N)	M/E series (N)	Mega-karyocytes (N)	Comments (N)	CFU-GM
3 ± 1	Control (10)	1:1 (1) 2:1 (5) 3:1 (3) 4:1 (1)	Complete (10)	None (5) Rare (5)	Rare mitotic figure (2) Hypocellular (1)	28.1 ± 4.5 to 232.2 ± 39.5
	Exposed (9)	2:1 (4) 3:1 (3) 4:1 (2)	Complete (8)	None (2) Rare (5)	Occasional mitotic figure (4) Inadequate cellularity (2)	9.8 ± 1.5 to 162.7 ± 2.7
10 ± 1	Control (10)	2:1 (7) 3:1 (2) 5:1 (1)	Complete (9)	None (3) Rare (7)	Rare mitotic figure (4)	11.7 ± 1.4 to 308.0 ± 38.7
	Exposed (9)	2:1 (5) 3:1 (3) 4:1 (1)	Complete (9)	None (2) Rare (7)	Occasional mitotic figure (4)	NFC ² to 228.0 ± 4.0
21 ± 1	Control (10)	1:1 (3) 2:1 (7)	Complete (10)	Rare (10)	Rare mitotic figure (4) Hypercellular (1)	36.7 ± 2.0 to 210.7 ± 25.4
	Exposed (9)	1:1 (4) 2:1 (5)	Complete (9)	None (2) Rare (7)	Occasional mitotic figure (4) Rare binucleated red cell (1)	14.3 ± 0.3 to 213.3 ± 53.5
30 ± 2	Control (9)	1:1 (5) 2:1 (2) 3:1 (1) 4:1 (1)	Complete (9)	None (4) Rare (5)	Rare mitotic figure (4) Binucleated red cell (1)	
	Exposed (9)	1:1 (3) 2:1 (6)	Complete (9)	None (2) Rare (7)	Occasional mitotic figure (4) Hypocellular (1)	

¹CFU-GM = colony forming unit—granulocyte-macrophage. Indicates range of colony growth per animal evaluated (mean ± standard deviation of enumerated colonies) with the lowest and highest number detected within each respective group represented.
²NFC = no formed colonies.

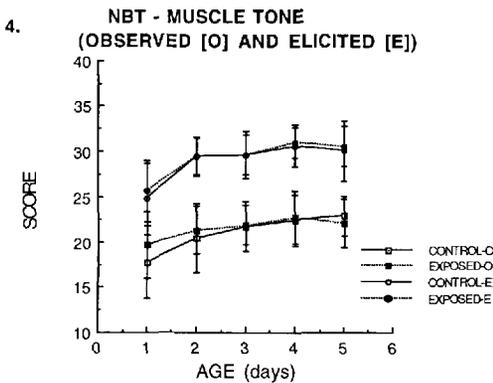


Fig. 4. Muscle tone for sham control and ultrasound-exposed infants during the first week of life as determined by the neurobehavioral test (NBT). There were no significant differences noted on any of the days evaluated; a marginal increase was observed for treated animals on day 1 only.

Human liver hematopoiesis begins at roughly 5 weeks gestation, with the bone marrow matrix (8–10 weeks), lymph nodes (11–12 weeks), spleen (8 weeks), and thymus (8 weeks) all functionally developing

thereafter (Kelemen et al., '79). Liver hematopoiesis is the major source of blood cells until roughly the end of the second trimester when the bone marrow takes over this function. Bone marrow hemopoiesis begins in different skeletal locations at varying times prenatally, and is uniquely dependent upon the course of ossification. In all parts of the developing skeleton that ossify by endochondral bone formation (i.e., cartilage model ossified to bone), it has been shown that cartilage disintegration is preceded by the immigration of a vascularized mesenchyme which eventually constitutes the framework upon which HSC proliferate. There is roughly a two week time lag between the development of this stromal matrix and the observation of the first extrasinusoidal hematopoietic islands within which bone marrow hemopoiesis begins (Flidner and Calvo, '78). Although little data is currently available which defines these events in the macaque, similarities in overall blood cell parameters prenatally (Tarantal, '92) in addition to other developmental features suggest that a comparable

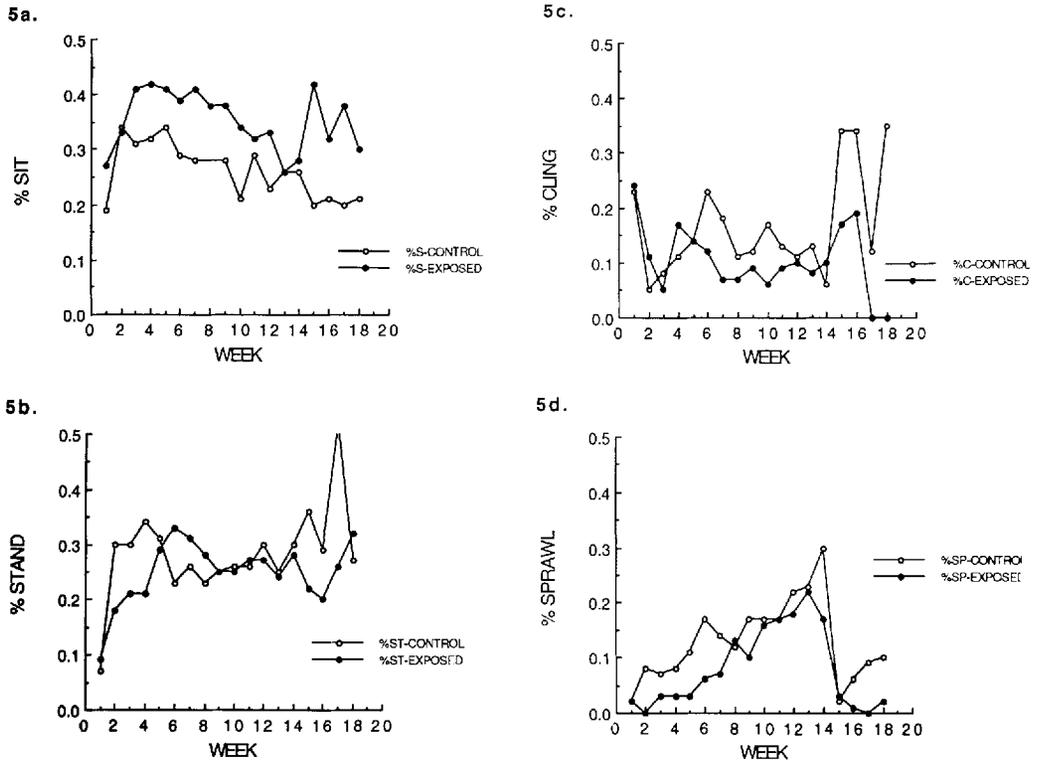


Fig. 5. Quiet activity patterns noted in an observation cage weekly from weeks 1–18. Note differences in patterns between control and exposed animals for % Sit (a), % Stand (b), % Cling (c), and % Sprawl (d). While infants exposed prenatally to ultrasound were sitting on

the bottom of the testing cage, controls were more actively engaged in climbing activities and in the resultant rest phases, namely clinging and sprawled along the sides of the testing apparatus.

course may be followed (Tanimura and Tanioka, '75).

In these studies, significant (yet transient) alterations in WBCs have been noted both pre and postnatally after frequent exposure to ultrasound prenatally. In an effort to further define the role of the progenitor cell in this effect, cultures of postnatal bone marrow aspirates with enumeration of colony growth were performed to assess myeloid lineage maturation. It was originally hypothesized that ultrasound exposure may produce a maturation arrest in the myeloid lineage by either directly inhibiting differentiation or by altering the supporting stroma. These affects were assumed to be related to a thermal mechanism, based on the concept that bone has a selective capacity for heat absorption due to its greater density (Tarantal and Hendrickx, '89b). Studies which evaluated the thermal rise

produced within the fetus (muscle/bone interface) by the ultrasound unit used in these studies have shown that the maximum elevation which can be achieved after 30 min exposure was 0.6°C (Tarantal et al., '92). Although this provides support for the premise that a temperature rise $\geq 1.0^{\circ}\text{C}$ would not be anticipated with this system, it has not been confirmed that elevations $\leq 1.0^{\circ}\text{C}$ are incapable of causing a biological effect that could be considered significant.

For the studies described, exposures were performed repeatedly throughout gestation, i.e., from early organogenesis through the end of the third trimester (GD 20–150). Although it is possible that frequent, short-term exposures could potentially provide a degree of protection (i.e., thermotolerance) (Mivechi and Li, '90; O'Hara et al., '91), it is also possible that a critical threshold could be reached whereby a switch from a poten-

tially protective environment to one that is potentially detrimental could occur. It is also plausible that the progressive nature of ossification (and hematopoiesis) within the developing skeleton or a particular event associated with development of the bone marrow during GD 120–140 could be critical in regard to the observed effect. It is interesting to note that there was no apparent effect detected in the bone marrow when collected postnatally since the assessment of CFU-GM colony growth and the myeloid precursor populations did not provide evidence for a reduction in the committed CFU-GM progenitors. Examination of stained bone marrow precursors also confirmed that there was no alteration in the M:E ratio or maturation of the myeloid lineage. The last prenatal ultrasound exposure performed on these animals was within two to four days prior to delivery and the first postnatal blood sample and bone marrow aspirate was collected two to three days thereafter. This provides roughly a one week period during which compensatory mechanisms would have to occur if the bone marrow was altered.

Another potential mechanism which could explain these effects is peripheral cell destruction, although there is no direct evidence that suggests such an event could have occurred. Since the fetuses and infants could not be classified as truly “neutropenic,” this may explain why there were no changes detected in the bone marrow. There are three primary factors that influence blood neutrophil concentration, namely (1) rate of input of neutrophils from the bone marrow storage pool to the blood, (2) rate at which cells are leaving the blood, and (3) proportion of cells that are circulating as compared with those in the marginated pool. This, therefore, implies a third possible mechanism which is that a greater population of neutrophils was marginated, which infers that there may have been a transient, benign impact on neutrophil kinetics. Approximately half of the neutrophils within the confines of the blood vessels are stuck to or rolling along the vessel walls (i.e., marginated); only half are circulating freely which indicates that the total number of neutrophils detected in a blood sample represents less than the actual number. Inflammatory stimuli, hemolysis, and hemodynamic abnormalities have been reported to lead to increased margination with a re-

sultant transient pseudoneutropenia (Beck, '87). Since pseudoneutropenia is not associated with an increase in the rate at which cells are entering the blood from the bone marrow, this could potentially explain the observed effects. Interestingly, *in vitro* studies have shown that high-power cavitation can produce hemolysis in whole blood (Miller, '88). It is difficult to extrapolate results obtained in an *in vitro* setting to *in vivo* conditions, although it has been suggested that effects that are readily noted *in vitro* may be subtle when diluted by large cell numbers such as under *in vivo* conditions.

There are few explanations that can be provided for the reduction in body weights and activity levels. It had originally been proposed that increased activity patterns *in utero* during ultrasound exposure could have resulted in offspring that were lighter due to decreased body fat, although not necessarily smaller (Tarantal and Hendrickx, '89a,b). As noted, decreased body weights after prenatal exposure have been reported in the literature in many of the species studied, although few have studied the potential mechanism(s) responsible. A study with gravid mice focused on maternal compromise as the primary mechanism for the birth weight changes (Barnett and Williams, '90). Both hind limb paralysis and dilated urinary bladders were noted consistently in these and prior studies with gravid mice which was hypothesized to be the result of a large area of the mouse abdomen exposure of to the ultrasound beam. Although it may be feasible for the mouse, maternal compromise as a mechanism is unlikely for the human and nonhuman primate, particularly since neither paralysis nor problems with micturition have ever been reported in either species, and the differences in size and surface area exposed during a typical examination precludes the possibility of an effect of this nature.

The alterations in activity patterns are an interesting observation, although it is unclear why the exposed animals would be more likely to sit quietly at the bottom of the testing cage when age-matched controls were more actively engaged (either standing, sprawled, or clinging to the side of the test cage). Although control infants were noted to be involved in activities that would appear to be more vigorous (i.e., standing or climbing), there were no appreciable differ-

ences detected in overall activity levels on a daily basis under typical housing conditions. It is, therefore, unlikely that these differences are suggestive of a significant biologic effect but may represent a subtle phenomenon.

Overall, the results of these studies have confirmed the presence of significant, yet transient, biologic effects in macaque offspring after frequent diagnostically-relevant ultrasound exposure in utero. The most significant findings are considered to be related to the potential alterations in hematopoiesis and reduced body weights, which may or may not be related. It is important to note, however, that all animals were able to compensate for these transient effects and appeared to remain unaffected by their occurrence later in life. As is typically the case, many questions still remain unanswered which implies that further investigations will be required in order to more fully identify the potential mechanism(s) responsible for these changes. It is possible that the observed hematologic effects may be due to heat and/or cavitation, although this will require a very defined effort in order to document *in vivo*. Those areas that are currently under investigation in this regard include: studies designed to determine whether a more acoustically challenging state-of-the-art commercial scanner (with "triple mode" capabilities) will exacerbate the identified effects while investigating the potential thermal rise generated with this system; evaluations which incorporate more frequent prenatal sampling in order to confirm that the prenatal effects described are restricted to a specific period of gestation (i.e., GD 120–140); and more challenging approaches such as the incorporation of *in utero* aspiration of fetal bone marrow in order to determine whether effects on progenitor cells can be detected prenatally.

At present, the FDA is considering revising its limitations on acoustic output of clinical scanners in an effort to eliminate imaging constraints on clinicians. This implies that an option for adding acoustic power to obtain clinically useful information will be available, which may also create a greater potential for biological interaction (i.e., heating, cavitation). The use of on-screen labelling has been proposed in order to provide a means whereby the potential for heating and acoustic cavitation can be mon-

itored with the use of a Thermal (TI) and Mechanical Index (MI), respectively (ODS, '92). With continued efforts towards identifying the interaction of ultrasound with the developing macaque fetus, one additional goal will be to correlate any significant findings with the TI and MI, and thereby attempt to identify the range in values that may be considered "safe" for human fetal exposure.

ACKNOWLEDGMENTS

This research was supported by NIH grants HD25528 and RR00169. The authors would like to thank Dr. Kevin Shannon for reviewing bone marrow slides and the manuscript, Dr. Esmail Zanjani and his laboratory staff for performing the stem cell assays, and the technical staff of the CRPRC Clinical Hematology Laboratory for their expert technical assistance, with a special thanks to Ms. Abigail Spinner.

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