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●*Original Contribution*

HEMATOLOGIC AND GROWTH-RELATED EFFECTS OF FREQUENT PRENATAL ULTRASOUND EXPOSURE IN THE LONG-TAILED MACAQUE (*MACACA FASCICULARIS*)

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Abstract—Prior investigations have shown that reduced birth weights and transient neutropenias result from frequent exposure of monkey fetuses to ultrasound. To further explore these findings, 26 animals were studied (16 exposed, 10 controls; "triple mode"; ATL Ultramark 9 with HDI®; I_{SPTAd} ~ 645 to 714 mW/cm²). Exposures were performed daily for 5 days each week from gestational days (GD) 21 to 35 (5 min), three times weekly from GD 36 to 60 (5 min), then weekly from GD 61 to 153 ± 1 (10 min). Fetal blood samples (FBS) were collected for complete blood counts (CBCs), hematopoietic progenitor assay, circulating insulin-like growth factors (IGF-I, IGF-II) and binding proteins (IGFBP-3) (GD 120, 140, 153 ± 1). Animals were delivered by Cesarean section at term (GD 153 ± 1), and body weights, morphometrics, CBCs, and bone marrow aspirates assessed at delivery and postnatally for 3 months. Fetal neutropenias were noted in exposed animals in addition to reduced circulating progenitors (colony forming unit–granulocyte-macrophage [CFU-GM]). Growth of CFU-GM from bone marrow was exuberant at term, whereas circulating levels were diminished comparable to prenatal samples. Exposed animals were smaller at birth; marked reductions in IGFBP-3 were noted prenatally. These data suggest that frequent prenatal ultrasound exposure can transiently alter the neutrophil lineage, although these findings may be the result of enhanced margination and organ sequestration. Data also suggest that transient, altered growth patterns may be due to perturbations of the IGF axis.

Key Words: **Bioeffects, Sonography, Monkey, Fetus, Hematopoiesis, Growth, IGF.**

INTRODUCTION

The obstetrical applications of ultrasound have expanded during the last four decades, as equipment design and sophisticated techniques for monitoring fetal development have evolved. With state-of-the-art imaging systems, fetal structures are more clearly defined, and techniques such as color flow imaging provide new ways for evaluating fetal status. Due to the capabilities of these systems, there is a tendency to conduct frequent evaluations for longer periods, thereby increasing fetal exposure. Although the FDA sets limits on the output of commercial scanners, the use of multiple imaging modalities has elevated these levels to the upper limit allowable. The recent adoption by the FDA (1993, 1994) of the output display standard (ODS) (AIUM/NEMA 1992) has resulted in the allowable limit for $I_{SPTA.3}$ to be increased from 94 mW/cm² for fetal applications to 720 mW/cm² for all applications. Although ultrasound is considered an appropriate method for obtaining information on fetal growth and development, it is acknowledged that there remain many gaps in our understanding of the interaction of this imaging modality with fetal tissues (AIUM 1993; Tarantal and O'Brien 1994). The fetus has been considered a special case when assessing safety issues since the length of the examination period, scanning techniques chosen, equipment used for the examination, experience of the operator and gestational age are all known to affect the "dose" received by the conceptus *in utero*. Of the various factors that have been considered in regard to safety, heating and cavitation remain of primary concern (AIUM 1993; WFUMB 1992).

The generation of heat during exposure to ultra-

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sound is a well-described phenomenon which is directly related to the propagation of sound energy while traveling through soft tissues. The percent of ultrasonic energy converted into heat via absorption is dependent upon the characteristics of the scanner and the tissue(s) under consideration (Lizzi and Ostromogilsky 1987; Nyborg and Steele 1983). Areas most likely to absorb ultrasonic energy are those directly associated with bone and at muscle/bone interfaces (Carstensen et al. 1990; Drewniak et al. 1989; Lehmann et al. 1967). The developing fetal skeleton presents a unique case due to increasing ossification throughout gestation. Although the general consensus is that the clinical application of diagnostic ultrasound is "safe" and poses no risk to mother or fetus, the pursuit of more information is clearly warranted due to the incompleteness of our understanding of biologic effects and the interaction of various imaging modalities with developing fetal tissues, and due to the recently adopted ODS (AIUM/NEMA 1992) by the FDA (1993, 1994) which allows for higher output levels.

The goals of these investigations were to: (1) further explore growth and hematopoietic changes documented in prior studies using a well-established fetal monkey model; and (2) compare findings from prior imaging studies with an older generation scanner (Tarantal and Hendrickx 1989a; Tarantal and Hendrickx 1989b; Tarantal et al. 1993b) to outcome after ultrasound exposure to a current state-of-the-art diagnostic imaging system.

MATERIALS AND METHODS

Animals

Normally cycling adult female long-tailed macaques (*Macaca fascicularis*) with a history of prior pregnancy were bred and identified as pregnant according to established methods (Tarantal and Hendrickx 1988a). All procedures employed within the study conformed to the requirements of the Animal Welfare Act, and study protocols were 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 California Regional Primate Research Center (CRPRC) operating procedures. Pregnancy in the long-tailed macaque is divided into trimesters by 55-day increments with gestational day (GD) 0 to 55 representing the first trimester, GD 56 to 110 representing the second and GD 111 to 165 representing the third (term ~ GD 165 ± 10).

Ultrasound system

The imaging system used for these studies was an Ultramark 9 with HDI® (Advanced Technology Laboratories [ATL], Inc., Bothell, WA). Output parameters were measured by the manufacturer, and are shown in Table 1. "Triple mode" settings (two-dimensional + pulsed and color Doppler) were incorporated, using either a linear array scanhead (L5; N = 10 exposed animals) or a curved array, intracavitary transducer (C9-5 ICT; N = 6 exposed animals) (see comments regarding animals, below). All exposures were performed transabdominally; animals were shaved prior to each exposure. A 1-mm Doppler sample volume (SV) was used with the smallest color box achievable under all conditions. The focal zone was set at either 1.2 cm (total depth setting of 2.5 to 3.5 cm; first and second trimesters) or 2.0 cm (depth settings of 3.5 to 4.5 cm; third trimester).

Experimental design

A total of 26 animals were studied, 16 exposed and 10 controls. Exposures were performed throughout gestation in hand-held, unanesthetized animals during GD 21 to 60 (daily for 5 days each week from GD 21 to 35, 3 times weekly from GD 36 to 60) for 5 min duration with the greatest length of the embryo imaged and the SV placed within the heart; and weekly from GD 61 to 153 ± 1 (near term) for a total of 10 min with the SV placed in the fetal heart for 5 min and then within the umbilical cord at the abdominal insertion site for 5 min. The last exposure was performed 20 min prior to Cesarean section on GD 153 ± 1.

Fetal blood samples (~2.5 mL) were collected by cardiocentesis using established methods (Tarantal 1993) in ketamine-sedated animals (10 mg/kg) on GD 120 and 140 for complete blood counts (CBCs) (0.3

Table 1. Operating condition quantities with the ATL HDI® system in triple mode.

Quantities	C9-5 ICT	C9-5 ICT	L5 Linear
Nominal focal death (cm)	1.2	2.0	2.0
Center frequency (MHz)			
Two-dimensional (2-D) echo	6.0	6.0	5.0
Color Doppler	5.0	5.0	4.0
Pulsed Doppler	5.0	5.0	4.0
Estimated source power (mW)			
2-D echo	0.34	0.62	1.19
Color Doppler	2.85	9.34	6.13
Pulsed Doppler	5.61	14.1	17.7
Footprint area (cm^2)			
2-D echo	1.23	1.23	2.30
Color Doppler	0.52	0.88	0.29
Pulsed Doppler	0.12	0.19	0.54
I_{SPTA} (mW/cm^2)			
Derated value	146	421	556
MI	1.0	0.9	1.5

I_{SPTA} = spatial peak, temporal average intensity using a derating factor of 0.3 dB/cm · MHz; MI = mechanical index.

to 0.5 mL), assessment of erythrocyte fragility (0.5 mL), hematopoietic progenitor assay (0.3 to 0.5 mL) and growth factor studies (0.7 to 1 mL). Samples were collected immediately after the exposures were performed. Samples for CBCs were placed directly into microtainer tubes with ethylene diaminetetraacetic acid (EDTA) (Becton-Dickinson, Rutherford, NJ). Parameters assessed included white blood cell counts (WBC), red blood cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet and reticulocyte counts, plasma protein and a differential. Slides were stained with Wright-Giemsa for morphologic evaluation. Samples for testing erythrocyte fragility were placed in microtainer tubes with lithium heparin (Becton-Dickinson), mixed for ~10 min, and 20-μL samples were added to varying concentrations of buffered saline solution (0.20% to 0.85%). Samples were incubated at room temperature (30 min), then centrifuged at 2000 rpm for 10 min. The amount of hemolysis in the supernatant was assessed with a spectrophotometer at 540 nm (Coleman Junior II, Model 6/20, Oak Brook, IL), with the optical density converted to the percentage hemolysis per tube.

For circulating hematopoietic progenitors, fetal blood samples were placed directly in sterile Iscove's modified Dulbecco's medium (IMDM) for culture according to established criteria, as previously described (Tarantal et al. 1993b). Briefly, 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% CO_2 in humidified air to determine the number and growth of colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), CFU mix and CFU–granulocyte-macrophage (CFU-GM) by colony formation in methylcellulose. Cells were evaluated after a standard 14-day incubation period. Smears were made for the determination of the relative percentages of immature and mature hematopoietic elements by direct examination of stained bone marrow (myeloid:erythroid [M:E] ratios). Smears were stained with Wright-Giemsa for morphologic evaluations.

Insulin-like growth factors (IGF-I, IGF-II) were evaluated in fetal and neonatal sera by radioimmunoassay (RIA), and IGF-binding proteins (IGFBP-3) were assayed then characterized by Western-ligand blot, according to established methods (Gargosky et al. 1992; Tarantal and Gargosky in press).

A Cesarean section was performed near term, and simian Apgar scores were assessed at 1, 5 and 10 min of life (Tarantal and Hendrickx 1989a). A complete physical and morphometric evaluation of the newborn was also conducted. Infants were nursery reared for the duration of the study (3 months). Body weights were evaluated daily and morphometrics weekly (crown–rump length; biparietal and occipitofrontal diameters, head and abdominal circumferences; hand, foot, humerus, and femur lengths; skinfold thicknesses), according to established methods which have been reported previously (Tarantal and Hendrickx 1989 a and b).

Blood (CBCs; 0.5 mL) and bone marrow aspirates (1 mL; progenitor assay, morphology) were collected at birth and on postnatal days 7, 21 and 30. Bone marrow was aspirated into heparinized syringes from alternating humeri using standard collection and assay techniques, as described above. Evaluations included quantitation of CFU-E, BFU-E, CFU mix and CFU-GM. M:E ratios were also assessed.

Calculated temperature increase

The monopole source solution was applied to a circular source to estimate the steady-state temperature increase (AIUM 1988, 1993; Ellis 1991; Ellis and O'Brien 1992, in press; NCRP 1992; Nyborg 1988; Pennes 1948). Input parameters for the monopole-source solution are the ultrasound frequency, the source aperture shape and size and the source power. The circular aperture slightly overestimates the temperature increase compared to a square aperture of the same aperture (footprint) area and same source power; the overestimation increases as a function of an increasing aspect ratio for a rectangular aperture of the same area (Ellis and O'Brien in press). An attenuation coefficient (and absorption coefficient) value of 0.5 dB/cm·MHz was used for a homogeneous tissue model to estimate the temperature increase *in vivo* since this value is more typical than that of soft tissue (NCRP 1992), whereas the ODS uses a value of 0.3 dB/cm·MHz (AIUM/NEMA 1992) to calculate the thermal index (TI) values. A moderate perfusion length value of 1 cm was used which was the same as that used in the ODS model. Axial profiles of the temperature increases were calculated under these conditions.

The ATL HDI® system operating in triple mode transmits three classes of pulses for each of the three imaging modes (Table 1). The monopole source solution was applied to each of these three classes of pulses (different frequencies, footprint areas and/or source powers) and yielded three separate axial temperature increase profiles (see Fig. 6) for one of the cases listed in Table 1 (C9-5, focal depth of 2.0 cm). The three individual axial temperature increase profiles (two-dimensional echo, color Doppler, pulsed Doppler) are summed to yield the calculated axial temperature profile for the combined three modes operating at the same

time to yield the estimated axial temperature increase profile (denoted combined in Fig. 6). The TI was also calculated according to the ODS procedures (AIUM/NEMA 1992) for the exposure conditions used herein.

Data analyses

All results (hematologic parameters, Apgar scores, morphometrics) were compared to concurrent and historical controls (N = 50). Group differences were assessed with repeated measures analyses (analysis of variance [ANOVA]) and Student's t test where appropriate. All statistical evaluations were performed on Apple Macintosh systems with statistical software (Statview 512+, Brainpower Inc., Calabasas, CA), and differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Overall, results with exposure via the linear or curved array were similar, and have, therefore, been grouped for all parameters.

There were no indications of gross malformations in exposed or control fetuses, similar to prior findings. There was one stillbirth and one premature delivery in the exposed group which was considered unrelated to the study. A nonsignificant increase in simian Apgar scores was noted in exposed animals (data not shown).

Fetal/neonatal hematologic studies indicated similar findings to those reported from prior investigations (Tarantal and Hendrickx 1989b; Tarantal et al. 1993b) (Fig. 1). Neutrophil counts were marginally reduced on GD 120 (designated as −32 days), significantly diminished on GD 140 (−12 days), elevated at term and then diminished thereafter. Growth of fetal blood samples in culture revealed significant reductions in CFU-GM on all days studied (GD 120, 140, 153 ± 1) (Fig. 2). Interestingly, correlative assay of bone marrow aspirates collected on the day of delivery showed exhuberant CFU-GM growth for exposed animals, with cell counts comparable to controls (Fig. 3). Postnatal evaluations indicated similar findings on day 7, with numbers roughly comparable to controls on day 30. Tests of erythrocyte fragility did not indicate any differences between the groups (data not shown).

Results of growth studies were more variable when compared to previous findings, although exposed animals were smaller at birth (Fig. 4). These differences were biologically relevant, but not statistically significant ($p > 0.05$). Assessment of circulating IGFs and IGFBP-3 indicated similar IGF-I and IGF-II levels, although marked reductions in IGFBP-3 were noted on GD 120 and 140 ($p < 0.05$), with increased values at term (GD 153 ± 1)(Fig. 5). IGFBP-3 levels were

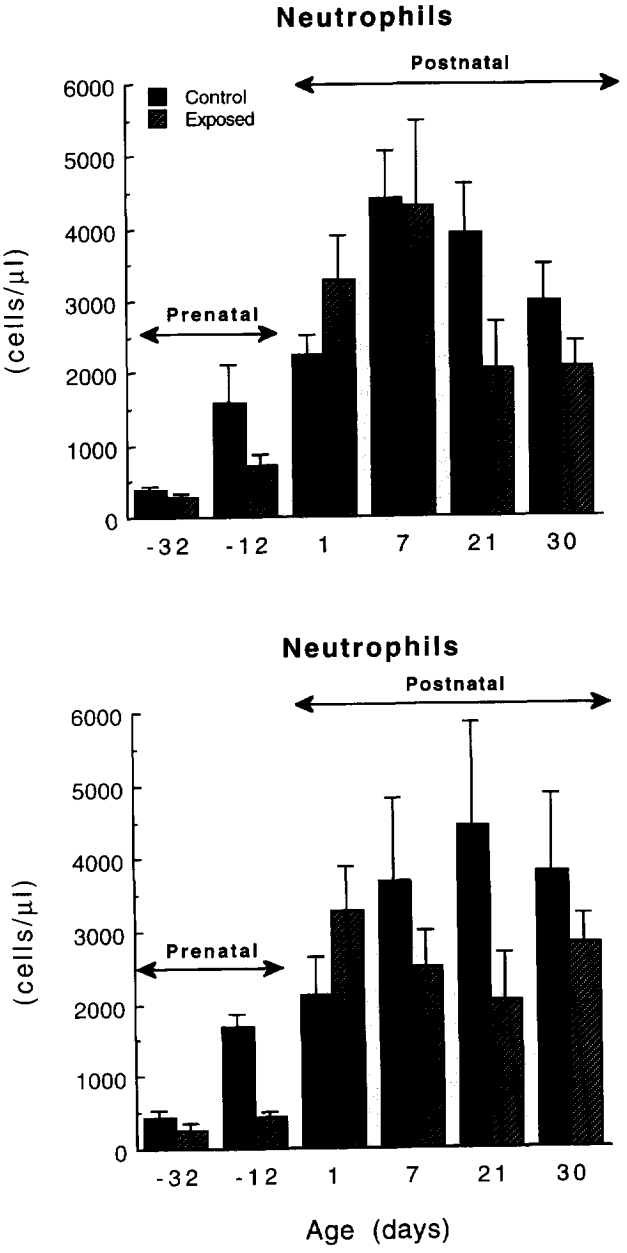

Fig. 1. (top) Neutrophil counts (cells/µL) for animals chronically exposed to ultrasound during gestation. Prenatal samples were collected on gestational day (GD) 120 (−32 days) and GD 140 (−12 days). (bottom) Comparison of results from studies described here to those reported from prior investigations with an older generation scanner (ATL, MK600, 7.5-MHz scanhead; two-dimensional imaging + pulsed Doppler; N = 25) (Tarantal et al. 1993b). Note similarities in outcome.

comparable between control and exposed animals on day 30 of life (data not shown).

Figure 7 shows the estimated axial temperature increase profiles for the three operating conditions

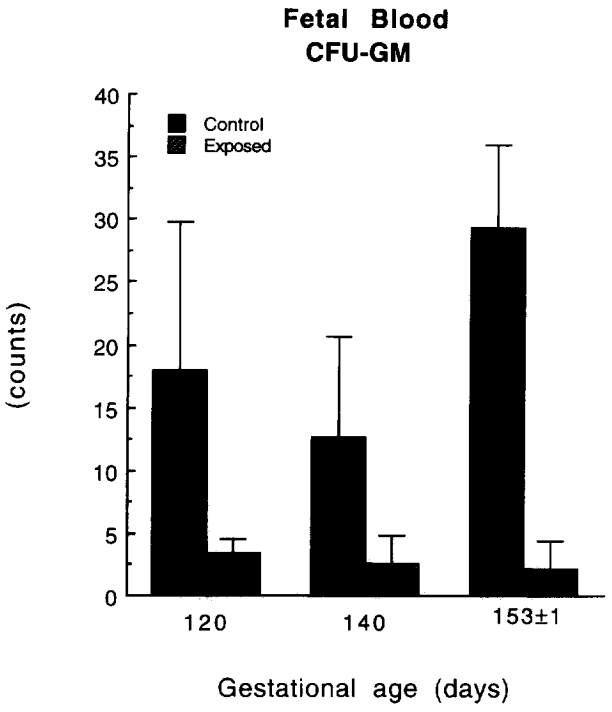

Fig. 2. Fetal blood samples collected by cardiocentesis and grown in culture for 14 days indicated significant reductions in colony forming unit–granulocyte-macrophage (CFU-GM) on all days studied. GD 153 ± 1 = day 1 or the day of delivery.

listed in Table 1—C9-5 (1.2), C9-5 (2.0) and L5 (2.0)—in addition to the axial temperature increase profile for the MK600 system/720A probe (2.2) reported in a previous study (Tarantal et al. 1993a). The maximum temperature increases for the C9-5 (1.2), C9-5 (2.0), L5 (2.0) and 720A (2.2) probe exposure conditions were, respectively, 0.28°, 0.52°, 0.48° and 0.27°C and the respective TIs for these four exposure conditions were calculated to be 0.21, 0.57, 0.46 and 0.34.

DISCUSSION

Blood cell formation in the fetus is a complex process, with the primary hematopoietic role gradually shifting from the liver to the bone marrow during the second trimester (Kelemen et al. 1979). The liver remains hematopoietic during prenatal life and through the first postnatal weeks, although the magnitude of its hematopoietic activity is considerably reduced during the third trimester and thereafter. Marrow hematopoiesis expands as liver and spleen decline, with ~65% to 70% of CFU-GM localized in the bone marrow during late gestation and the early postnatal stages (Tavassoli and Yoffey 1983). Effective hematopoiesis is a multistep process consisting of stem cell proliferation and cell maintenance, differentiation into committed cell populations, orderly maturation into functional cells and transport into the circulation, depending on body demands (Beck 1987).

Within this cascade are potential explanations for the ultrasound-induced changes observed in monkey fetuses chronically exposed to ultrasound *in utero.* There may be a transient decrease in production of CFU-GM, defective maturation of this differentiated population, or sequestration of cells within the hematopoietic tissues (liver and/or bone marrow), perhaps as a result of induced cell adhesion. Any of these changes could affect both mature (neutrophil) and immature (CFU-GM) cell populations in the peripheral circulation. Correlative evaluation of colony growth from blood and bone marrow near term indicate exhuberant CFU-GM growth in bone marrow, while CFU-GM in peripheral blood substantially diminished. This could indicate a compensatory response in the bone marrow, or could imply effects specific to the peripheral circulation. We have previously reported that margination and/or increased destruction of formed elements may occur peripherally (Tarantal et al. 1993b). It is reasonable to consider that the CFU-GM population may be reduced as a result of heating since other laboratories have shown that CFU-GM are uniquely sensitive to heat when compared to other progenitor populations

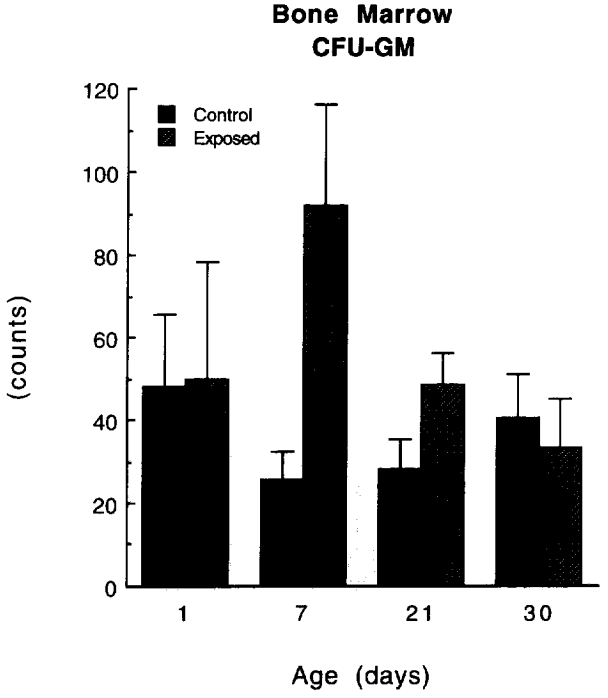

Fig. 3. Bone marrow aspirates grown in culture from infants delivered near term (day of delivery = day 1), then days 7, 21 and 30. Numbers normalized to control values by day 30.

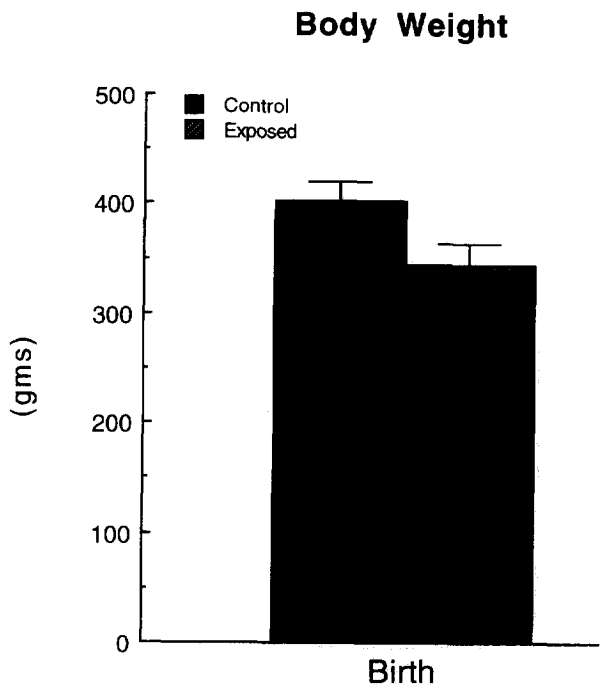

Fig. 4. Birth weights for control and ultrasound-exposed newborns. Differences were biologically but not statistically different.

(Mivechi 1988; Mivechi and Li 1990). It is proposed that the changes observed are the result of altered hemodynamics and transient inflammation due to tissue heating, both of which can induce cell adhesion. Neutrophils constitutively express cell adhesion molecules (CAMs) and can rapidly alter their number or functional state in response to these stimuli (Zimmerman et al. 1994). This process can be rapidly reversed or even sustained for hours depending upon the nature and intensity of the stimulus. It is also reasonable to consider that an increase in production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) may occur. TNF-α is known to promote margination by increasing expression of vascular endothelial adhesion molecules. Although we have no current evidence to support that these events occur as a result of frequent ultrasound exposure during the third trimester, reports have indicated that human fetal Kupffer cells (tissue macrophages that line hepatic sinusoids) secrete TNF-α and interleukin (IL)-1β when stimulated (Kutteh et al. 1991). Both IL-1 and IL-6 are known to induce synthesis of acute phase proteins in fetal hepatocytes as a result of inflammation or injury (Bauer et al. 1991). Further studies on margination and the role of enhanced cytokine production and CAM expression will be required in order to explore these hypotheses.

Reduced birth and body weights have been reported as a result of ultrasound exposure in multiple animal models in addition to the human (Miller and Ziskin 1989; NCRP 1992; Newnham et al. 1993; Tarantal and Hendrickx 1989a; Tarantal and Hendrickx 1989b; Tarantal et al. 1993), although it is currently unclear how ultrasound can affect the growth process.

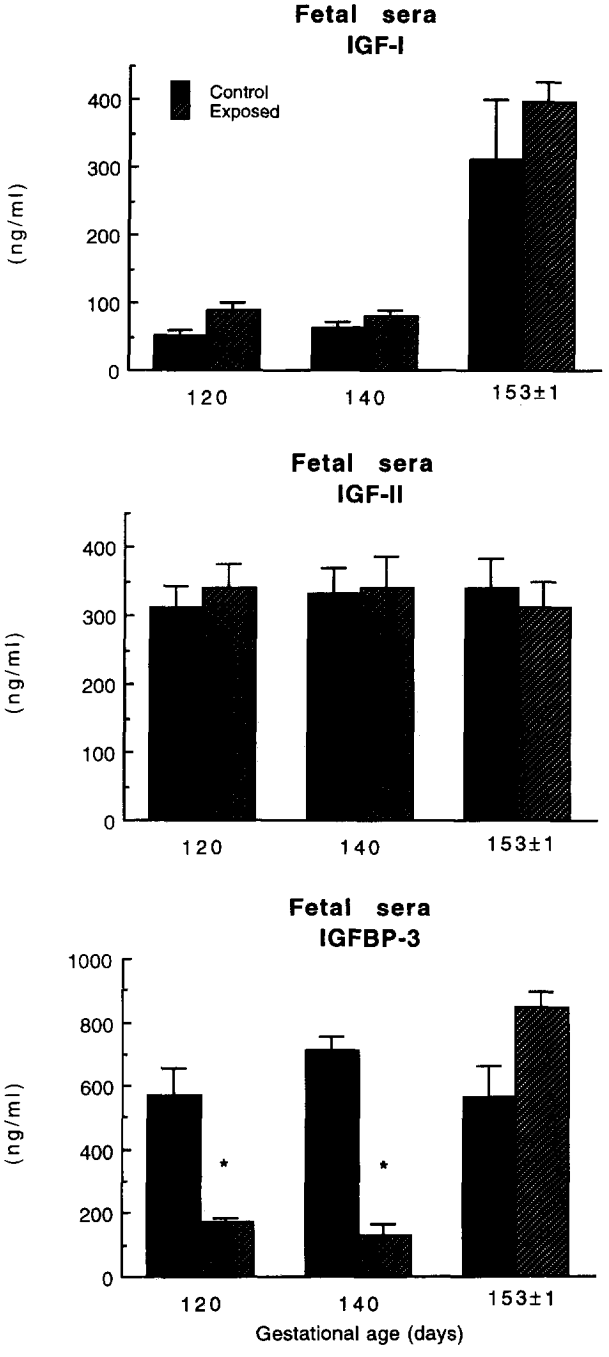

Fig. 5. No change in circulating IGF-I (top) or IGF-II (middle) were detected in exposed fetuses, although significant reductions in IGFBP-3 (bottom) were detected. Values normalized by day 30 of life (data not shown).

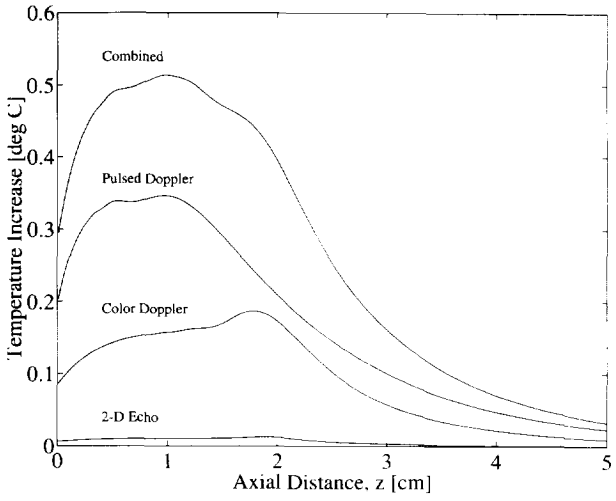

Fig. 6. Axial temperature increase profiles using a homogeneous soft tissue model (attenuation and absorption coefficient = 0.5 dB/cm·MHz; perfusion length = 1 cm) for the C9-5 ICT (2.0 focal depth) probe values listed in Table 1. The combined axial temperature increase profile (top curve) is the sum of the three axial temperature increase profiles.

Since the IGFs are known to play a central role in fetal growth (DeChiara et al. 1990), evaluations of these important mitogenic and differentiative factors may provide important insights into bioeffects mechanisms. In the mammalian fetus, circulating concentrations of IGF increase during gestation, although only a small percentage of the circulating IGFs are free in the circulation (unbound) due to high concentrations of IGFBPs (Holly and Wass 1989). In the intrauterine growth restricted (IUGR) human fetus, IGFBP-3 has been shown to be substantially reduced (roughly 30%) when compared to normal birthweight babies (Giudice et al. in press). Interestingly, monkey studies have also shown that chronically exposed fetuses have decreased circulating IGFBP-3, which may be modulated at the transcriptional or translational level. Proteolysis of IGFBP-3 (a major serum carrier of IGF) is thought to provide a control mechanism whereby IGFBP-3 levels decline while the availability of IGF to target tissues increases, possibly during stages of accelerated fetal growth (Lamson et al. 1993; Lassarre and Binoux 1994; Suikkari and Baxter 1992). It is possible that changes in IGFBP-3 may be compensatory, and thereby protect the exposed fetus from severe growth alterations, although the primary mechanism(s) for these changes are currently unknown. It is important to note that growth factors such as IGF-I and -II and cytokines such as TNF-α and the interleukins form a large family of extracellular signaling molecules with very similar mechanisms of action on both hematopoiesis and growth. A role for TNF-α in IUGR has also been proposed (Schiff et al. 1994), and interactions between the IGFs and TNF-α involving local IGFBP regulation have been shown (Martin and Baxter 1991; Yateman et al. 1993). TNF-α has been reported to reduce the endocrine action of the IGFs by altering IGFBP-3 secretion which could imply an important role for TNF-α during chronic ultrasound exposure. Since immunocytochemical studies have identified the liver as a major site of IGF production (Han et al. 1988), and IGFBP-3 is synthesized primarily in hepatic parenchymal cells, ultrasound-induced hepatic effects may be a significant event.

In conclusion, studies with the chronically exposed fetal monkey have further supported our findings of transient effects on body weights and the neutrophil lineage, with evidence indicating that the third trimester is the most susceptible to these effects. It is proposed that similar changes could potentially occur in the human fetus, but remain undetected due to their transient nature, the limited sampling of fetuses during the periods identified as susceptible, and because no significant changes would be anticipated postnatally. It is important to note that we have not, to date, identified any long-term ramifications of these events, as all infants studied under all exposure conditions have resumed a normal postnatal developmental course. Continued studies with relevant animal models such as the monkey will help to further our understanding of potential prenatal biologic effects, periods of devel-

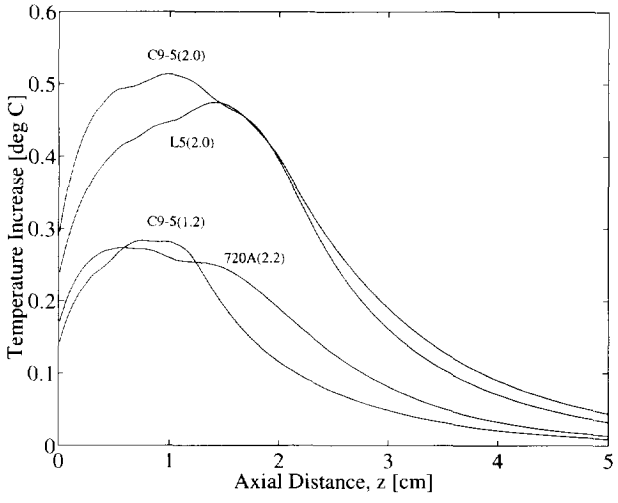

Fig. 7. Combined axial temperature increase profiles using a homogeneous soft tissue model (attenuation and absorption coefficient = 0.5 dB/cm·MHz; perfusion length = 1 cm) for the operating conditions listed in Table 1 (C9-5 probe at nominal focal lengths of 1.2 and 2.0 cm and L5 probe at 2.0 cm) in addition to the axial temperature increase profile for the 720A probe at the nominal focal length of 2.2 cm used in a prior study (Tarantal et al. 1993a).

opment and organ systems that may be sensitive to frequent exposure, and the mechanism(s) responsible for their occurrence.

SUMMARY

Studies have shown that transient reductions in body weights and third trimester neutrophil counts can occur in the monkey fetus and neonate as a result of frequent exposure to ultrasound prenatally. Evidence suggests these effects are induced at the organ-specific (liver, bone marrow) and peripheral level, may be due to heating, and that the third trimester is the most sensitive time period. Results also indicate that effects on growth patterns may be due to perturbations of the IGF axis, with reductions in IGFBP-3 potentially providing a compensatory mechanism for preventing significant growth dysregulation.

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