Decreased bone ultrasound velocity in premature infants conceived with assisted reproduction

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Quantitative ultrasound (QUS) measurement of tibial speed of sound (SOS) can be used to determine bone strength. Children conceived with assisted reproduction treatments (ART) are taller than naturally conceived (NC) children, so we hypothesized that these infants would have higher SOS levels than NC infants. Thirty-seven ART (ART group) and 51 NC neonates (NC group) were included in the study. Tibial initial SOS (iSOS) was measured within 96 hours of birth using QUS. Measurements were performed weekly until the infant was discharged from the hospital. The iSOS levels of the ART group (2823.41±110.8 m/sec) were lower than those of the NC group (2917.14±145.6 m/sec) ($p=0.001$). A decrease in SOS levels was observed in 39 of 53 infants who had serial scans. The difference in SOS levels between the first and last scan was significant ($p<0.02$). In vitro manipulation during the periconceptual period can result in metabolic alterations in bone mineral content. Contrary to our null hypothesis, bone SOS of infants in the ART group was found to be lower than in the NC group. Moreover, bone SOS decreases in early postnatal life. This result emphasizes the fact that even with advances in nutritional care, the ex utero environment remains a poor substitute for in utero development.

***Key words:** neonate, speed of sound, quantitative ultrasound, assisted reproduction, bone.*

Despite advances in neonatal care, osteopenia, characterized by skeletal demineralization and fractures that can occur during normal handling, remains a common and potentially serious complication of prematurity[1]. Preterm infants have increased risk of low bone mass and a greater need for bone nutrients because of limited accretion of bone mass in utero[2,3].

Poor bone health in preterm infants is due to the combination of reduced mineralization and low bone mass, and its assessment remains difficult[4]. Although the combination of low serum phosphate and high alkaline phosphatase markedly improves sensitivity, this is seen late in the course of the disease, and the diagnosis of osteopenia remains difficult[5]. Plain X-rays, in the absence of fractures, are a poor diagnostic method.

The measurement of bone mineralization in neonates has represented a methodological challenge. Several different techniques have been used to measure the biophysical properties of bone, such as single- or double-beam photon absorptiometry or dual energy X-ray absorptiometry, which require expensive equipment and also expose patients to some degree of radiation[6].

Quantitative ultrasound (QUS) is a noninvasive, radiation-free, portable and relatively inexpensive technique that measures the speed of sound (SOS) of an ultrasound signal between the two transmitters and receivers within a probe. QUS measures bone density in addition to cortical thickness, elasticity and microarchitecture, therefore providing better and accurate measurements of bone strength[7,8]. Several reports have recently suggested QUS measurement of bone speed of sound as an important tool for diagnosis and follow-up of bone strength in premature infants with osteopenia[3,4,9,10].

We[11] and others[9-14] have successfully used QUS (Sunlight Medical Ltd, Tel Aviv, Israel) to measure tibial SOS in sick preterm infants cared for in their incubators, without the need for sedation. We recently published two separate bone SOS curves, which were made for Turkish neonates born of appropriate-for-gestational-age (AGA) single and multiple pregnancies of 25-41 weeks gestation[11], and our previous data suggests that QUS can be safely used to accurately determine bone status in neonates as early as the first day of life. However, there are no data on SOS measurements in prematurely born infants conceived with assisted reproduction treatments (ART), namely in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). These infants are taller than naturally conceived (NC) children, and it is speculated that genomic imprinting may contribute to the taller stature of IVF children[15]. Therefore, we hypothesized that prematurely born ART infants might have higher bone SOS values than NC infants. The aim of the present study was to find out whether ART infants have an advantage over NC infants in bone strength and to investigate the relationship between prenatal risk factors, perinatal morbidities and SOS levels in these infants.

Material and Methods

Subjects

Eighty-eight appropriate-for-gestational-age neonates observed in the NICU of Hacettepe University Faculty of Medicine, whose gestational ages ranged between 24 and 34 weeks and birth weights between 670 and 2680 grams, were included in the study. Gestational age was determined by the first day of the last menstrual period, intrauterine ultrasound dating or the Ballard scoring system[16] at birth. Neonates were divided into ART and NC groups. Premature infants born via assisted reproduction treatments (IVF and ICSI) were designated as the ART group, and other infants, born of naturally conceived pregnancies, were designated as the NC group.

Cases with congenital diseases, chromosomal abnormalities or malformations, intrauterine infections, neuromuscular disorders and multiple pregnancies, along with small- or large-for-gestational-age infants, were excluded. No infants had renal or endocrine disease, growth retardation or malabsorption.

Data collection

At the time of each ultrasound measurement, data on the infant's current clinical status [respiratory distress syndrome (RDS), patent ductus arteriosus (PDA), necrotizing enterocolitis (NEC), pneumonia, sepsis, indirect hyperbilirubinemia (IHB)], together with demographic data, were recorded. The number of days on total parenteral nutrition (TPN) and the total calories of enteral and parenteral feeding during the study period were recorded daily. Five of the premature infants had bronchopulmonary dysplasia and received systemic steroids and diuretic treatment; they were excluded from the study. No infant had a clinically evident fracture during the study period. On the basis of climate statistics and changes in air temperature, spring here refers to March, April and May; summer, to June, July and August; autumn, to September, October and November; and winter, to December, January and February. History of continuous usage of a headscarf during pregnancy for religious reasons was also recorded.

Nutritional management

Total parenteral nutrition was initiated in the first 24 hours of age with subsequent increases providing up to 3.5-4 g protein/kg/day and 2-3 g lipid/kg/day. 1.5 mL/kg Cernevit® (Baxter, Heidelberg, Germany) was added to the lipid solution, and 2.32 mEq/kg/day calcium and 1.5-2 mmol/kg/day phosphorus were added to TPN. Minimal enteral nutrition was initiated when infants achieved hemodynamic stability and was advanced as tolerated by 10-30 ml/kg/day according to the gestational age of the infant. Full enteral feeding was considered to be 150 ml/kg/day. Vitamin D (400 IU/day) was added for all premature infants receiving enteral feeding starting from the second postnatal week. Infants were fed either with fortified breast milk or with preterm special formula (Prematil-LCP®, Milupa, Friedrichsdorf, Germany).

Ultrasound scans

Quantitative ultrasound (Sunlight Omnisense

Table I. Demographic Characteristics, Prenatal, Natal and Postnatal Risk Factors, and SOS Levels of ART and NC Groups

	NC Group	ART Group	p
Number of cases (%)	51 (57.9)	37 (42.1)	
Gestational age at birth (wk) (mean±sd)	30.5±1.9	30.0±1.9	>0.05
Birth weight (g) (mean±sd)	1437±500	1364±306	>0.05
Male:female	26:25	18:19	>0.05
Initial SOS (m/s) (mean±sd)	2917.1±145.6	2823.4±110.8	<0.001
ΔSOS (m/s) (mean±sd)	24.6±133.4	47.8±122.2	>0.05
History of PROM, n (%)	14 (27.4)	7 (18.9)	>0.05
Use of headscarf, n (%)	6 (11.7)	7 (18.9)	>0.05
Cesarean section, n (%)	37 (72.5)	31 (83.7)	>0.05
Preeclampsia, n (%)	14 (27.4)	5 (13.5)	>0.05
Gestational diabetes, n (%)	2 (3.9)	4 (10.8)	>0.05
RDS, n (%)	17 (33.3)	23 (62.1)	<0.01
PDA, n (%)	7 (13.7)	9 (24.3)	>0.05
NEK, n (%)	1 (1.9)	1 (2.7)	>0.05
Pneumonia, n (%)	5 (9.8)	4 (10.8)	>0.05
Sepsis, n (%)	15 (29.4)	11 (29.7)	>0.05
IHB, n (%)	22 (43.1)	25 (73.5)	>0.05
Age at initial enteral feeding (d), median	2 (1-3)	2 (1-3)	>0.05
Age at full enteral feeding (d), mean	8.24±6.3	9.24±5.9	>0.05
TPN duration (d), median	4 (0-27)	4 (1-24)	>0.05

PROM: premature rupture of membrane, RDS: respiratory distress syndrome, PDA: patent ductus arteriosus, NEC: necrotizing enterocolitis, IHB: indirect hyperbilirubinemia, TPN: total parenteral nutrition

Premier 7000P, Tel Aviv, Israel) was used to measure bone SOS. The mean of three measurements of tibial SOS was used for data analysis. All measurements were made within the first 96 hours of birth and weekly thereafter by the same person.

Delta SOS (ΔSOS) was used to describe the difference in SOS levels between the first and the last measurement.

The Hacettepe University Human Ethics Committee approved the study (No. FON 03/37-10) and written informed consent was obtained from at least one of the parents of each infant included in the study.

Statistical analysis

Data were analyzed with the SPSS 11.5 statistical package (SPSS, Chicago, IL, USA). Results are expressed as mean ± SD, or as median with minimum and maximum levels when data were not homogeneously distributed. The distribution of the data was assessed using a one-sample Kolmogorov–Smirnov test. To compare means among groups, an independent or dependent sample t-test or Mann–Whitney U test was performed for homogeneously and nonhomogeneously distributed data, respectively. The chi-square test was used for the comparison of categorical parameters; in instances where it was not appropriate, Fisher's exact test was used. The level of significance was set at p<0.05.

Results

Of the 88 infants included in the study, 37 (42.1%) were in the ART group and 51 (57.9%) in the NC group. Demographic characteristics, prenatal, natal and postnatal risk factors, and SOS levels of the ART and NC groups are displayed in Table I.

The mean initial SOS (iSOS) levels of the ART group (2823.4±110.8 m/sec) were significantly lower than those of the NC group (2917.1±145.6 m/sec) (p<0.001).

In the ART group, iSOS levels were positively correlated with both gestational age at delivery and birth weight (p=<0.001, r=0.565 and

Table II. Effect of Various Prenatal Risk Factors on Mean Initial SOS Levels

Prenatal risk factors		NC Group (n=51)			ART Group (n=37)			P**
		n	iSOS (mean±sd)	p*	n	iSOS (mean±sd)	p*	
Gender	Female	27	2931.7±131.3	>0.05	18	2837.0±128.8	>0.05	0.02
	Male	24	2908.5±160.2		19	2810.4±92.3		0.02
Preeclampsia	Yes	14	2963.1±107.3	>0.05	4	2860.5±68.9	>0.05	0.05
	No	37	2904.1±155.2		33	2818.8±114.8		0.01
Diabetes	Yes	2	3019.5±116.7	>0.05	4	2753.7±111.6	>0.05	0.05
	No	49	2916.5±145.4		33	2831.8±109.4		0.006
Use of headscarf	Yes	6	2924.3±170.2	>0.05	7	2787.3±138.3	>0.05	>0.05
	No	45	2920.1±143.3		30	2831.8±104.4		0.005

p*: indicates the significance of the difference between the subgroups (gender, preeclampsia, diabetes and headscarf)
P**: indicates the significance of the difference between the NC and ART groups

Table III. Relation of ΔSOS and Various Perinatal Morbidities

		NC Group (n=31)			ART Group (n=22)		
		n	ΔSOS (mean±sd)	p	n	ΔSOS (mean±sd)	p
RDS	Yes	11	83.3±139.7	>0.05	12	68.4±86.6	>0.05
	No	20	54.3±167.9		10	60.8±90.7	
PDA	Yes	5	115.6±55.2	>0.05	7	72.1±65.3	>0.05
	No	26	54.8±168.4		15	54.9±96.8	
Sepsis	Yes	11	41.2±192.5	>0.05	7	78.0±68.9	>0.05
	No	20	73.0±135.2		15	52.2±95.1	
IHB	Yes	16	70.2±79.9	>0.05	17	8.8±95.8	>0.05
	No	15	58.6±213.8		5	75.5±80.7	

RDS: respiratory distress syndrome, PDA: patent ductus arteriosus, NEC: necrotizing enterocolitis, IHB: indirect hyperbilirubinemia

p=0.019, r=0.382, respectively), whereas in the NC group, iSOS levels were correlated only with gestational age at delivery (p=0.019, r=0.331). There was no significant difference in the iSOS levels of the ART and NC groups according to gender, presence of preeclampsia or diabetes in the mother, or headscarf usage by the mother (Table II). However, when we subdivide the neonates according to these variables (gender, preeclampsia, diabetes and headscarf usage), all of the neonates in the NC group had higher iSOS levels than those in the ART group, although the differences in the subgroups did not reach statistically significant levels due to the low number of subjects in these groups. The effects of various prenatal risk factors on mean iSOS levels are given in Table II.

When the effect of various neonatal morbidities on ΔSOS levels was investigated, the change in SOS levels (ΔSOS levels) did not differ between the groups and subgroups (Table III).

Additionally, the iSOS levels of the neonates did not differ according to the seasons in either the NC or the ART group (Table IV).

Fifty-three infants had serial scans (22 infants in the ART group and 31 infants in the NC group). Overall, the median number of measurements was 2 (range: 2-5). SOS levels decreased in 39 (73.6%) of these 53 infants [22 (56.4 %) in the NC group and 17 (43.6%) in the ART group, p=0.60]. The difference in SOS levels between the first and last scan was significant (p<0.02). However, there was no difference in ΔSOS levels of the ART and NC groups.

When we evaluate the 53 infants who had serial SOS measurements, feeding characteristics did not differ between the infants whose SOS levels decreased and those whose SOS levels increased (Table V). However, the birth weight and gestational age of those infants whose SOS levels decreased were significantly lower (Table V).

Of the 88 infants included in the study, 74 received TPN. There was no significant correlation between age of initial enteral feeding (p= 0.313, r=0.144), TPN duration (p=0.365, r=0.128) and total daily caloric intake and ΔSOS values (p=0.595, r=-0.075). There was a statistically significant mild to moderate positive correlation between day of full enteral nutrition achievement and ΔSOS values (p=0.021, r=0.336).

Discussion

Assisted reproduction treatments, namely IVF and ICSI, have become widely used in the treatment of infertility. It is well established that assisted reproduction is associated with adverse perinatal outcomes, including increased risks of preterm delivery, low birthweight and increased neonatal mortality[17-19]. The growth of IVF children has been of concern for the last two decades. One of the major topics of follow-up in these children has been their height[20]. Taller stature was also shown in IVF very low birthweight infants, suggesting that the mechanism was operative before or during conception or in early gestation[21]. The authors postulated that ART children might be displaying subtle alterations in DNA methylation patterns in imprinted genes that are more markedly associated with growth. There have been a few QUS studies of bone status in Turkish newborns, but no data exists concerning SOS values in prematurely born ART infants.

The present study demonstrates that QUS assessment of tibial SOS can be performed successfully in premature infants whose gestational ages are less than 34 weeks and therefore can serve as a useful tool to estimate bone strength in premature infants. The possible reasons for lower bone SOS and marked decrease in bone SOS after birth in premature infants are: (a) premature infants' calcium and phosphorous reserves are not sufficient, and the provision of calcium and phosphorous after birth is inadequate in comparison with that which takes place in the intrauterine environment[3,22]; (b) premature infants have lower bone loading—the mechanical catalyst that influences the process of bone formation and resorption[23]. There was a significant correlation between the SOS levels and birth weight and gestational age measured in the first week of life in this study, similar to what has been reported in previous studies, reflecting the rapid rate of bone mineral accrual during the last trimester[10,14,24-25].

Table IV. Comparison of Bone iSOS (m/s) of Infants Born in Different Seasons

	NC Group				ART Group			
Season	n	iSOS (mean±sd)	Comparison	p	n	iSOS (mean±sd)	Comparison	p
Spring	14	2921.5±121.2	Spring vs. summer	>0.05	9	2813.6±75.8	Spring vs. summer	>0.05
			Spring vs. autumn	>0.05			Spring vs. autumn	>0.05
			Spring vs. winter	>0.05			Spring vs. winter	>0.05
Summer	12	2895.1±162.8	Summer vs. autumn	>0.05	9	2817.1±109.8	Summer vs. autumn	>0.05
			Summer vs. winter	>0.05			Summer vs. winter	>0.05
Autumn	17	2916.5±170.1	Autumn vs. winter	>0.05	9	2862.0±119.1	Autumn vs. winter	>0.05
Winter	7	2972.4±95.8			10	2803.0±136.4		

We have shown that bone SOS of ART neonates is lower than that of NC neonates. These results are contrary to our null hypothesis, which was that bone SOS of ART neonates would be higher than that of NC neonates. The mechanism causing tall stature in IVF children is poorly understood[20]. Insulin-like growth factor 2 (IGF-2) is an important factor influencing fetal growth during the last trimester[26]. Serum levels of growth-stimulating hormones such as insulin-like growth factor 1 (IGF-1), IGF-binding protein 3 and IGF-2 were seen to be higher in ART infants than in NC infants. An epigenetic mechanism during the IVF procedure has been suggested[27]; indeed, livestock have large offspring after in vitro culture, which was associated with aberrant methylation and expression of the imprinted IGF-2 receptor gene [20]. Recently, a New Zealand study[15] found that a cohort of 69 IVF and ICSI children aged between 4 and 10 years (mean age, 5.9 years) were significantly taller than 71 NC controls, after adjustment for age and parental height. They also reported higher serum concentrations of IGF-1 and -2 in the IVF and ICSI children and concluded that the observed differences in stature and growth factor may be due to subtle epigenetic alterations of imprinted genes as a result of the IVF process. ART children were exposed to estrogen and progestatives early during implantation[28]. Estrogen lowers the mechanostat strain set point on endosteal bone surfaces, which causes cortical thickening through increased endocortical bone accrual[29,30]. For these reasons, we speculated that ART infants would have higher SOS values at birth than other infants. However, the low SOS values of ART infants observed in this study did not support our hypothesis. Genomic imprinting has been increasingly recognized as a key determinant of intrauterine growth[27]. Since its potential association with IVF-related problems has become a topic of major interest, some imprinted genes have been found to have important roles in embryonic/fetal growth[31,32]. While the reason is unclear, we found significantly lower bone SOS levels in ART infants born prematurely; we suggest that our findings may represent epigenetic alteration of imprinted or nonimprinted genes. Moreover, the possibility that the genetic predisposition underlying infertility results in an increased frequency of imprinting defects has been suggested[33]. Indeed, imprinting defects and infertility may have a common cause, and this may further increase the risk of conceiving a neonate with an imprinting defect[34].

Seasonal variation in serum 25(OH)D is a well-established phenomenon, which is due to reduction in UVB penetration through the atmosphere during the winter months when the sun's zenith angle is increased[35]. Because pregnancy traverses several seasons, it is evident that maternal status at a given point in pregnancy is influenced by the season of measurement as well as other environmental and physiological factors[36]. Namgung et al.[37,38] reported marked seasonal differences in bone mineral content (BMC) in neonates. Summer-born infants had significantly lower BMC and significantly higher cord serum osteocalcin and $1,25(OH)_2D3$ concentrations than did winter-born infants. However, our SOS results failed to agree with findings of BMC seasonal trends; there was no significant difference in SOS levels between the various seasons.

Turks have argued about the permissibility of veiling for over a decade[39]. Erkal et al.[40]

Table V. Feeding Characteristics, Gestational Age and Birth Weight of Infants Who Had Serial SOS Measurements

	Decreased SOS	Increased SOS	p
Number of cases (%)	39 (73.6)	14 (26.4)	0.60
Gestational age at birth (wk) (mean±sd)	30.03±1.9	31.3±1.6	0.028
Birth weight (g) (mean±sd)	1222±276	1673±468	0.001
Age at initial enteral feeding (d), median (25-75%)	2 (2-2)	2 (1-2)	0.26
Age at full enteral feeding (d), median (25-75%)	4 (4-6)	4 (4-4)	0.48
TPN duration (d), median (25-75%)	3 (2-4)	3 (2-4)	0.39

found wearing a scarf to be an independent risk factor for vitamin D deficiency in Turkish women living in Turkey and Germany. The efficiency and amount of ultraviolet B-induced production of vitamin D depends on the dose of relevant wavelengths of ultraviolent radiation to precursors in the skin[41]. Women pregnant at high-altitude locations, during winter or effectively sun protected, e.g., darkly pigmented and/or veiled, are at increased risk of vitamin D insufficiency, the latter even in high ambient ultraviolent radiation environments[42,43].

In this study, 13 of the mothers wore a headscarf constantly, for religious reasons. We found no significant differences in bone SOS measurements between infants of women who did and did not wear a headscarf. However, we did not measure maternal or cord serum concentration of vitamin D; this is a limitation of our study. We therefore cannot make valid inferences about the effect of maternal headscarf use on bone SOS measurements.

Severe morbidity during the neonatal period also increases the risk of bone demineralization in premature infants[44]. Development of bronchopulmonary dysplasia, chronic treatment with diuretics and steroids, the need for total parenteral nutrition and prolonged immobility further increase the risk of bone demineralization[10]. However, we found no difference in ΔSOS values between various perinatal morbidities (respiratory distress syndrome, patent ductus arteriosus, necrotizing enterocolitis, pneumonia, sepsis and indirect hyperbilirubinemia) in the present study.

Bone mineralization is modulated by genetic, nutritional, hormonal and mechanical factors[45]. However, since the major cause of osteopenia in premature infants is inadequate postnatal calcium and phosphorus intake, most therapeutic efforts to prevent osteopenia of prematurity have focused on nutritional changes[44]. Yet despite optimal support for growth in premature infants (which results in growth similar to that in utero during the last trimester), nutritional interventions have been only partially successful in improving such infants' bone mineralization[46]. Delayed or inadequate enteral feeding with disturbed mineral metabolism due to dependence on TPN may lead to poor linear growth and fractures in the preterm infant[45]. SOS values correlate negatively with duration of TPN[47].

However, we found no significant correlation between ΔSOS levels and day of initial enteral nutrition, TPN duration and daily caloric intake. There was significant correlation only between day of full enteral nutrition achievement and ΔSOS levels ($p=0.021$, $r=0.336$). The earlier the infant achieved full enteral nutrition, the less the drop in SOS level. Therefore, we may conclude that early and aggressive enteral feeding might lower the incidence of osteopenia of prematurity.

Previous reports have shown that bone SOS decreases in the early postnatal life of premature infants[48-50]. Based on these findings, we performed serial QUS measurements in 53 infants. SOS levels decreased in 39 (73.6%) of these infants. The reason for the decrease in postnatal SOS is not yet fully understood. In fetal life, the loading of the fetal skeleton arises from movement against the resistance of the uterine muscle, such as when the fetus kicks against the uterine wall. Furthermore, the fetus is exposed to high placental estrogen levels during intrauterine life[45]. Following birth, there is unloading of the skeleton because of the cessation of movement against resistance, which, along with the loss of placental estrogen, leads to endocortical bone resorption and hence thinning of the cortex. Therefore, one reason for the postnatal decline in SOS levels observed in our study might be the cortical thinning following endocortical bone resorption, as previously reported by Beyers et al.[51]. Another reason might be the increase in weight and subsequent changes in body mass index[13]. The bone mineral density of long bones decreases by about 30% in the first months after birth, reflecting a redistribution of bone tissue from the endocortical to the periosteal surface[52]. Tomlinson et al.[50] recently suggested that this decrease may be attributed to higher morbidity or to nutritional factors. This indicates that even with advances in neonatal nutritional care, which have been primarily focused on improving bone mineral accretion, the ex utero environment remains a poor substitute for the uterus in terms of nurturing fetal development. Our data are in agreement with those of Litmanovitz et al.[9] and Tomlinson et al.[50], who reported declining serial tibial SOS measurements in the first 8 weeks of age. There was a significant decrease in SOS values over time. These results indicate that despite adequate nutrition, there is a significant decrease in bone strength during early postnatal life.

In conclusion, the increasing survival rates for ART preterm infants necessitate the development of improved methods to assess bone health in this vulnerable population. QUS is a useful tool for documenting bone status in these infants. This is the first study showing that bone SOS of ART infants is lower than that of NC infants. This may be due to subtle epigenetic alteration of imprinted genes or to other genes that undergo epigenetic modification and are involved in growth and bone status. The reason for the lower SOS levels in the ART group could not be clarified in this study. Further studies are needed to explain this outcome, evaluating in particular the effects of medications given to the mother for induction of pregnancy on the development of bones in the fetal period. We have also shown that bone SOS shows a decreasing trend in the early postnatal life of premature infants. This emphasizes the fact that even with advances in neonatal nutritional care, the ex utero environment remains a poor substitute for in utero development.

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