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Tactile/kinesthetic stimulation (TKS) increases tibial speed of sound and urinary osteocalcin (U-MidOC and unOC) in premature infants (29–32 weeks PMA)

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ABSTRACT

Preterm delivery (<37 weeks post-menstrual age) is associated with suboptimal bone mass. We hypothesized that tactile/kinesthetic stimulation (TKS), a form of infant massage that incorporates kinesthetic movement, would increase bone strength and markers of bone accretion in preterm infants. Preterm, AGA infants (29–32 weeks) were randomly assigned to TKS ($N=20$) or Control ($N=20$). Twice daily TKS was provided 6 days per week for 2 weeks. Control infants received the same care without TKS treatment. Treatment was masked to parents, health care providers, and study personnel. Baseline and week two measures were collected for tibial speed of sound (tSOS, m/sec), a surrogate for bone strength, by quantitative ultrasound (Sunlight8000) and urine markers of bone metabolism, pyridinium crosslinks and osteocalcin (U-MidOC and unOC). Infant characteristics at birth and study entry as well as energy/nutrient intake were similar between TKS and Control. TKS intervention attenuated the decrease in tSOS observed in Control infants ($p<0.05$). Urinary pyridinium crosslinks decreased over time in both TKS and CTL ($p<0.005$). TKS infants experienced greater increases in urinary osteocalcin (U-MidOC, $p<0.001$ and unOC, $p<0.05$). We conclude that TKS improves bone strength in premature infants by attenuating the decrease that normally follows preterm birth. Further, biomarkers of bone metabolism suggest a modification in bone turnover in TKS infants in favor of bone accretion. Taken together, we speculate that TKS improves bone mineralization.

Introduction

Premature birth results in impaired bone mineralization because the majority (~80%) of fetal bone mineralization occurs in the last trimester of pregnancy [1]. Despite major advances in neonatal nutritional supplementation, bone mineralization of preterm infants does not increase as it would *in utero* [2,3]. This results in impaired bone integrity that can range from mild undermineralization to rickets [1]. Poor bone mineralization has been found to persist in prematurely born children and young adults [2,3]. Evidence suggests a resultant reduction in peak bone mass, weaker bones, shorter stature, and a higher rate of fracture in children born prematurely compared to full term peers [4–6]. Most research has focused on very low birth weight (VLBW) or premature infants born before 29 weeks. However, children (3–5 years) born prematurely in the last trimester (30–37 weeks gestation) also exhibit smaller bones and lower bone mineralization compared to term children [7]. This is an important population to consider because infants born in the last trimester represent over 90% of all U.S. preterm births (<37 weeks) [8].

Tactile/kinesthetic stimulation (TKS), a form of infant massage that includes kinesthetic movement, is promoted as an effective intervention to enhance postnatal bone development in premature infants. Premature birth exposes infants to stressful stimuli and immobilization, both of which are associated with poor bone mineralization. Both stress and movement are critical issues in preterm infant care. Premature infants admitted to the newborn intensive care (NICU) are exposed to many care giving activities and procedures which increase activation of stress systems. Stress hormones released chronically in response to physical and environmental stressors are associated with reduced growth and bone mineralization in prematurely born children and adults [9]. Preterm infant care also reduces kinesthetic stimulation in a number of ways. Movement occurs with little resistance, tactile stimulation is avoided to reduce disruptions to the infant, and drugs used to reduce pain decrease muscular activation. Absence of kinesthetic stimulation is considered a significant contributor to decreased bone mineralization in preterm infants [10]. Multiple investigators have reported on the impact of TKS in the form of infant massage and kinesthetic movement separately and together as one modality. Evidence from these studies

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suggests improved stress response [11,12] from massage and a positive impact of kinesthetic movement on bone mineralization particularly in VLBW infants [13–16].

Quantitative ultrasound (QUS) measurement of bone speed of sound (SOS) provides a highly reproducible and noninvasive assessment of bone properties to assess the impact of TKS in the preterm infant [17]. SOS is a proxy measure for bone strength and provides information on bone mineralization and density, cortical thickness, elasticity, and microarchitecture [18,19]. Previous studies demonstrate that tibial SOS is an effective means to assess bone status and have consistently detected a postnatal decline in SOS in preterm infants [20–24]. SOS assessment of the impact of kinesthetic movement in VLBW preterm infants has found attenuation of this decline in this population [13,14].

Bone is a dynamic tissue which is continuously subjected to resorption and formation. In normal homeostasis, bone metabolism is in balance to maintain the mass and microarchitecture of the skeleton. Quantitative changes in bone metabolism can be assessed by measuring biochemical markers of bone remodeling in serum or urine. Biochemical markers of bone resorption and formation could thus provide insight into the mechanistic effects of TKS on bone development. Serum is difficult to collect in preterm infants, particularly for longitudinal studies, but many bio-markers can also be detected in urine. Specific biochemical markers for bone resorption, pyridinium crosslinks of collagen (Deoxypyridinoline (Dpd) and Pyridinoline (Pyd)) allow assessment of bone resorption by osteoclasts in urine samples. The carboxylated form of osteocalcin (OC) binds with high affinity to hydroxyapatite of bone. In circulation, osteocalcin is, in general, considered a marker of bone formation by osteoblasts and serum osteocalcin is positively correlated with bone mineral density (BMD) in adults [25–27]. Urine osteocalcin midfragments (U-MidOC) that accumulate in urine serve primarily as an index of basal bone turnover. In a study of elderly females, U-MidOC correlated with markers of resorption and formation (e.g. serum CTX, TRACP5b, and BoneALP) [28]. U-MidOC has also been identified as a predictor of catch-up growth in infancy in preterm infants [29]. The undercarboxylated form of osteocalcin (unOC) is released into circulation during bone turnover and has been recognized for its ability to increase insulin sensitivity by increasing beta-cell production of insulin and adipose tissue release of adiponectin in mice [30,31]. Little is currently known about the endocrine action of uncarboxylated osteocalcin in early life.

The aim of the present study was to investigate, prospectively, TKS as a therapy to improve bone strength and biochemical markers of bone development in preterm infants. We hypothesize that twice daily TKS therapy improves bone modeling (assessed with biochemical markers of bone turnover) and results in greater bone strength. Appropriately grown preterm infants born in the last trimester (29 to 32 week PMA) were targeted. In addition to biochemical markers of bone development, this study includes measures of unOC. Results will help to assess the use of TKS as a complementary therapy to benefit early bone development in preterm infants.

Methods

A prospective, masked, longitudinal study design was used in which preterm infants were randomized to daily TKS or Control. Dietary intake was tracked daily. Anthropometry, quantitative ultrasound (QUS) measurement of bone speed of sound (SOS), and urine and blood spot samples were collected at study start and at 2 weeks.

Subjects

Preterm infants were recruited for study from the neonatal intensive care unit at the University of Utah Hospital or Intermountain Medical Center, Salt Lake City, Utah. Recruitment was limited to 28 4/7–32 3/7 weeks post-menstrual age (PMA) confirmed by physical exam at birth, maternal dates, and mid-pregnancy 2-D fetal ultrasound. Criteria for inclusion included: medically stable infants appropriately grown for gestational age (5th to 95th percentile) tolerating enteral feeding of 100 ml/kg/d by day of life 14. Exclusion criteria included birth injury, congenital anomalies, severe CNS injury (birth asphyxia, congenital hydrocephalus), complex cardiac defects, hypothyroid, or inborn errors of metabolism as these types of conditions may influence the infant's development. Infants began the study protocol when tolerating enteral feeding volumes of $\geq$100 ml/kg/day.

The study complies with the World Medical Association Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of the University of Utah. Written informed consent was obtained from the parents. At the time of informed parental consent, infants were stratified by gender (male, female) and feeding (human milk versus premature infant formula) and randomized to TKS or control by random draw from an envelope.

Intervention

TKS was performed by a licensed massage therapist for 20 minutes twice daily, 6 days a week, for Study Days 1–15. The data present results from a 2-week duration. TKS consisted of the application of six moderate strokes to the infant placed in a supine position in the following areas: 1) legs from top of thighs to ankles and feet, 2) chest over ribcage, 3) shoulders to hands, 4) head from crown to neck and including face, 5) back from neck to waist (performed with infant remaining in supine position) [32]. This method is modeled after Infant Massage USA's protocol but modified for preterm infants and to include kinesthetic movement to the arms and legs. During the kinesthetic movement, the infant continues in a supine position and each arm and then each leg moved away (extension) from mid-line against the infant's own resistance and back (flexion) with resistance from the therapist. Extension/flexion movements at ankles, knees, hips, wrists, elbows, and shoulders are repeated 5 times for each arm and each leg as previously described [15,16,33]. Control infants are placed in a supine position without tactile stimulation and without kinesthetic movement. Control is also performed by a licensed massage therapist for 20 minutes twice daily on Study Days 1–15. TKS or Control treatment assignment was masked to other study personnel, parents, and NICU clinical staff. The massage therapist remained behind a privacy screen for 20 minutes to maintain masking. Only the massage therapist and the PI were aware of the infant's study assignment to minimize bias by caregivers and study personnel during data collection.

Speed of sound

The left tibial speed of sound (SOS) was measured by Quantitative Ultrasound (QUS, Sunlight Omnisense 8000S with the PREMIER software). QUS operates on the axiom that the speed of sound (SOS) is directly proportional to bone density. The speed at which sound propagates through the bone is calculated as the time between sound transmission and receipt. System quality verification was performed daily, using a manufacturer supplied phantom made from a stable material through which sound waves propagate at known speeds. Tibial SOS was measured in the same leg as tibia length measures. SOS of the left tibia was done at mid-shaft, defined as half way between the base of the foot while flexed at a 90 degree angle to the tibia and the top of the knee. The point of measurement was marked perpendicularly to the direction of the bone. Three to five consecutive measurement cycles were performed, after which SOS (m/s) was determined. Technicians performed two series of measurement cycles on each limb to ensure precise results. The final result was reported as the average of the two measurements.

Urinary biomarkers of bone metabolism

Bone turnover was assessed in urine samples. Urine samples were collected over 6 hr (0900 to 1500 hr) into a sterile urine collection bag, weekly during either TKS or Control intervention and stored at −70 °C. Quantitation of collagen degradation products excreted in the urine (urinary pyridinium crosslinks) was used to identify levels of bone resorption [34]. Urinary pyridinium crosslinks, Deoxypyridinoline (Dpd) and Pyridinoline (Pyd), were analyzed using HPLC analysis (ARUP Laboratories, University of Utah). Osteocalcin midfragments (U-MidOC) were detected by a previously described two-site immunoassay based on monoclonal antibodies 6F9 and 3H8 [28]. Undercarboxylated osteocalcin (unOC or Glu-OC), the metabolically active form of osteocalcin, was detected in urine samples with an EIA kit (Takara Bio Inc.). Performance of unOC EIA with urine has been assessed and is an approved specimen type. All assay results were normalized to urinary creatinine levels measured by Quantitative Spectrophotometry. All measures were performed blind and in duplicate.

Statistical analysis

Comparisons were made by two-way ANOVA with treatment and time as independent variables. Differences between means were analyzed with post-hoc analysis using Fisher's least significant difference. A p-value <0.05 was considered statistically significant. Statistical analysis was carried out with SPSS® version 17.0 (SPSS Inc., Chicago, IL, USA). A Monte Carlo simulation process was done to estimate power using Stata v9 statistical software using variance estimates from a previous study. A sample size of 36 infants (18 in each treatment group) was determined to provide sufficient power (at least 80%).

Results

Characteristics of subjects and nutritional management

Informed parental consent was obtained for 51 infants. Eleven infants (4 TKS and 7 Control) had incomplete data collection due to hospital transfer or discharge before day 15 of study ($n=9$) or parental withdrawal of consent ($n=2$). Data collection was complete for two consecutive weeks for 40 infants (20 TKS, 20 Control). Infants were medically stable and receiving full enteral feedings. Gender and ethnic distribution as well as gestational age, body weight, and body length at birth were similar for TKS and Control infants. The amount of increase in weight gain, body length, and tibia length were also similar over the length of the study period (Table 1). The average energy and protein as well as calcium, phosphorus, and vitamin D intake for the entire study period ($\leq$ 28 days) was similar between TKS and Control infants (Table 2).

Quantitative ultrasound measurements of tibial SOS

Tibial speed of sound (tSOS), a reflection of cortical thickness and bone mineral density, was decreased in control (ANOVA; $p<0.05$). TKS intervention attenuated the rate of decrease in tSOS seen in control infants (Fig. 1). There were no significant differences in baseline tSOS between the two groups. Further, TKS infants had similar tSOS at week two compared to baseline. However, TKS tSOS was significantly higher than controls (Fisher's LSD post-hoc analysis; $p<0.05$) by week two.

Urinary markers of bone metabolism

Measures of markers for bone resorption, urinary pyridinium crosslinks, Dpd and Pyd, were similar between groups (Table 3). There was a trend for increased Pyd from baseline to week two (ANOVA, $p=0.08$). Differences between Dpd and Pyd from baseline to week two were analyzed using paired t-test. The differences were similar between groups ($p=0.9$; $p=0.2$, respectively). The Dpd/Pyd ratio, indicative of bone turnover, decreased significantly by week two in both control and TKS infants (Fig. 2; ANOVA, $p<0.005$). Urinary osteocalcin midfragment (U-MidOC) was significantly increased after 2 weeks of intervention (ANOVA, $p<0.001$). Particularly, U-MidOC levels increased in TKS group from baseline to week two (Fig. 3; interaction effect $p<0.05$ and Fisher's LSD post-hoc analysis; $p<0.001$) whereas no similar increase was observed in the Control group. Further, U-MidOC was 32% greater in TKS infants when compared to control infants by week two (Fisher's LSD post-hoc analysis; $p=0.01$). Urine measures of the undercarboxylated form of osteocalcin (unOC) were increased in TKS infants from baseline to week two (ANOVA and Fisher's LSD post-hoc analysis; $p<0.05$). This resulted in a trend toward increased unOC levels ($p=0.07$) compared to the control group at week two (Fig. 4).

Table 1
Characteristics of the study participants.*

	Control infants ($n=20$)	TKS infants ($n=20$)
Gestational age (weeks)	31 ± 0.9	31 ± 0.8
Postnatal age (weeks)	32 ± 0.1	33 ± 1.0
Gender (female/male)	9/11	9/11
Ethnicity (Hispanic/non-Hispanic)	3/17	8/13
Birth weight (g)	1574 ± 246	1632 ± 238
Birth length (cm)	41 ± 2	41 ± 2
Weight gain (g/kg/day)	15.7 ± 4	16.4 ± 3
Length increase (cm/day)	0.14 ± 0.06	0.14 ± 0.09
Left tibial length increase (cm/day)	0.07 ± 0.01	0.06 ± 0.04

Mean ± SD; *No statistically significant differences.

Discussion

Our findings support the hypothesis that twice daily TKS administered to premature infants in the neonatal period improves bone strength as assessed by tibial speed of sound. Tibial speed of sound (tSOS), a reflection of cortical thickness and bone mineral density, decreases briefly after birth [20–24]. The decrease in tSOS is thought to result from a thinning of tibial cortical bone due to postnatal endocortical bone resorption. Consistent with results from VLBW infants, we found that TKS in appropriately grown preterm infants born in the last trimester experienced attenuation in the rate of decrease of tSOS. Furthermore, biomarkers of bone metabolism suggest a modification in bone turnover in TKS infants in favor of bone accretion.

Elevated U-MidOC and unOC in TKS infants appear to reflect an overall increase in bone accretion because of maintained bone strength. However, it is important to note that a negative association between unOC and bone mineral density in postmenopausal women has been observed [35]. Further, inhibitors of bone resorption decrease serum unOC in patients with osteoporosis [36]. Overall, unOC is associated with bone turnover markers in adults and decreases with corticosteroid treatments [37,38]. However, the associations of unOC with resorption in early development may be different than in adults. At early age, bone metabolism is mostly bone modeling and the balance is in favor of bone formation, whereas in adults bone remodeling predominates.

Table 2
Nutrient intake during study.*

	Control infants ($n=20$)	TKS infants ($n=20$)
Fluid intake (ml/kg/day)	142.9 ± 10	141.1 ± 11
Energy (kcal/kg/day)	110.0 ± 8	108.0 ± 9
Protein intake (g/kg/day)	3.19 ± 0.4	3.20 ± 0.4
Calcium intake (mg/kg/day)	210 ± 19	203 ± 19
Phosphorus intake (mg/kg/day)	114 ± 10	110 ± 10
Vitamin D intake (IU/day)	283 ± 22	273 ± 25

Mean ± SD; *No statistically significant differences.

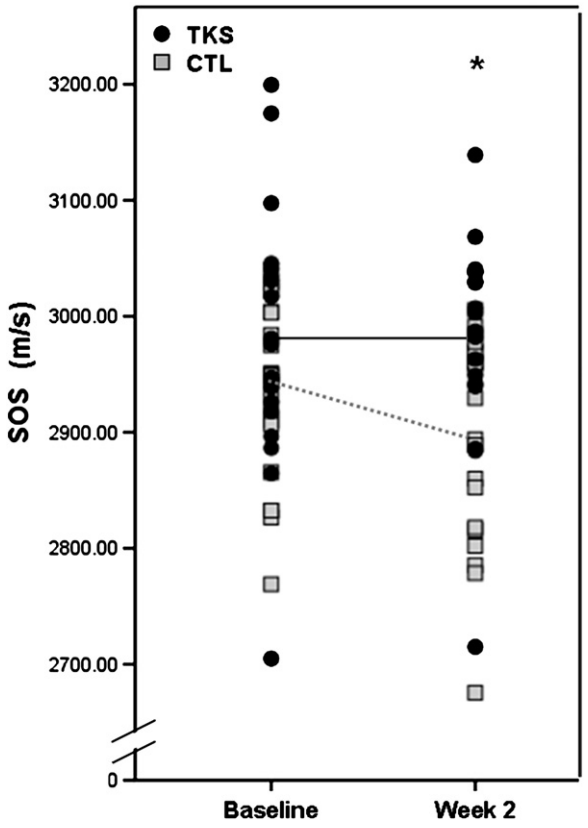

Fig. 1. Tibial speed of sound (tSOS). During the study period, tSOS decreased in control infants. The tSOS of TKS and Control (CTL) was significantly different by week 2. (*$p<0.05$).

Elevated U-MidOC and unOC in TKS infants may also have metabolic implications. The urinary marker of bone metabolism, U-MidOC, predicts catch-up growth in preterm and term infants out to 1 year of age [29]. Accelerated, or 'catch-up', postnatal growth has both positive and negative consequences. A lack of catch-up growth results in neurodevelopmental problems and stunted growth while infants who experience catch-up growth have elevated risk of adult metabolic abnormalities [39]. TKS has consistently resulted in improved neurodevelopment [40–43] but the impact of TKS on metabolism has not been adequately addressed. Studies suggest that infant massage increases weight gain [43] and Field et al. reports increased insulin in preterm infants after only 5 days of massage intervention [44]. Massage is generally thought to increase weight gain, although there are studies reporting no change in weight including present study [16,40,45]. The primary concern is with regard to growth quality, including lean mass accumulation and location of fat deposition, beyond weight gain. Preterm infants experience a dysregulation of glucose metabolism [46,47]. Bone is an endocrine organ that influences insulin sensitivity via the release of unOC [48]. Increased unOC is linked to increased glucose tolerance and enhanced insulin sensitivity, although human clinical evidence of this is limited [49]. The endocrine impact of increased bone metabolism and release of unOC during early life on the development of adult disease is unknown. Improved bone strength (tSOS) and the possibility of improved insulin sensitivity, mediated in part by increased unOC, indicate an immediate positive impact from TKS treatment. The long-term impact is an intriguing area for future research in the developmental origins of disease. To our knowledge, this is the first report of unOC in preterm infants.

Table 3
Urine pyridinoline crosslinks.*

	CTL	TKS
Pyridinoline (Pyd)		
Baseline	857 ± 327	932 ± 262
Week 2	940 ± 273	1133 ± 317
Deoxypyridinoline (Dpd)		
Baseline	222 ± 93	244 ± 87
Week 2	237 ± 113	261 ± 71

Mean ± SD; μmol/mol creatinine; *No statistically significant differences.

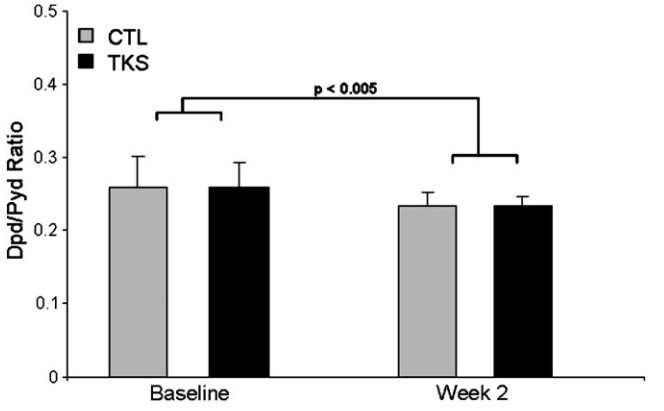

Fig. 2. Dpd/Pyd ratio. Control (CTL) and TKS infants decreased Dpd/Pyd ratio over the study period (mean ± SD).

One major etiological factor that contributes to subnormal bone mineralization in preterm infants is the deficiency of substrate (e.g. Ca and P) to match intrauterine accretion rates of bone mineralization. Increased Ca and P in parenteral nutrition helps attenuate the decrease in SOS detected in preterm infants [50]. However, despite supplementation of sufficient amounts of Ca and P, bone mineralization remains low in preterm infants. Dietary intake of calcium and phosphorus was similar between TKS and control infants in this study and met current advised intake levels. Vitamin D is also a key player in bone mineralization. The AAP recommends a vitamin D supplementation of 400 IU/day. Standard protocol is to provide infants 200 IU/day supplemental vitamin D in addition to the dietary intake reported in Table 2. TKS is hypothesized to improve growth via the effects on vagal tone and gastric motility to improve nutrient absorption that may also benefit bone mineralization [51].

Stress hormones have a well described negative impact on bone mineralization. We have previously reported that TKS treatment improved autonomic nervous system balance [32]. Furthermore, evidence from clinical studies suggests that massage can decrease hormonal markers for stress such as cortisol and epinephrine [11,52–56]. Levels

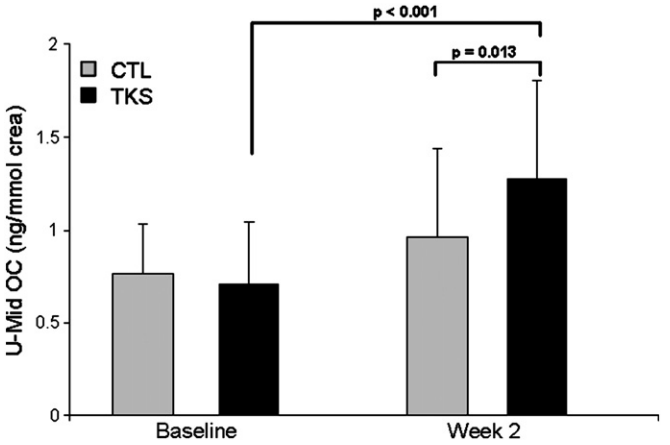

Fig. 3. Urinary osteocalcin midfragments (U-MidOC). TKS increased U-MidOC from baseline to week 2 and had a significantly higher level at week 2 compared to control (CTL). Mean ± SD.

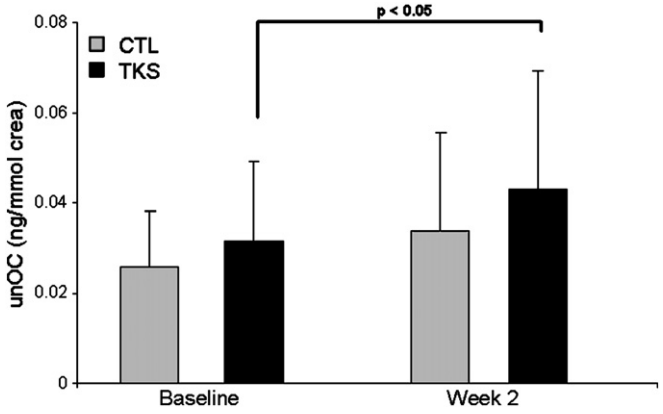

Fig. 4. Urinary undercarboxylated osteocalcin (unOC). TKS infants had increased unOC from baseline to week 2. Mean ± SD.

of chronic stress hormones are negatively associated with growth quality as a result of growth hormone suppression and development of insulin-like growth factor 1 (IGF1) resistance [57]. Chronic stress also suppresses osteoblastic activity responsible for bone formation [57,58]. This can be seen in children with anxiety disorders who have shorter stature, slower growth, and increased risk of osteoporosis later in life [59]. Animal models of tactile stimulation and maternal care also demonstrate improved stress response with enhanced endogenous pain control due to increased opioid and oxytocin expression, improved ANS tone, and increased release of gastric hormones and growth factors [60–62]. The improved stress response appears to be maintained into adulthood. Adult rats given TKS like treatment in the neonatal period have lower corticosterone release in response to stressors [63]. Furthermore, our group has shown improved bone phenotype and changes in fat deposition that spans into adolescent life as a result of neonatal TKS treatments in rat models [64–66].

Mechanical loading on bones and joints enhances bone formation and immobilization results in increased bone resorption and decreased bone mass in children and adults [67–71]. For example, absence or limited mechanical loading or weight-bearing activity is strongly associated with bone resorption as seen in immobilized adults (e.g. paraplegia) [70,71]. Kinesthetic movement has been shown to reduce the risk of bone loss and fractures in children, young adults, and older individuals. Improved physiologic stability, greater fat-free mass, and increased bone mineralization and bone strength in premature infants receiving a kinesthetic movement protocol compared to infants who received routine care have also been reported [14–16,33,72]. Aly et al. report that kinesthetic movement with massage resulted in approximately a 50% increase in biomarkers for bone formation in preterm infants but there were no measures of bone phenotype [73]. Our results demonstrate a similar attenuation in tSOS as those reported in VLBW infants but to a lesser degree. The degree to which preterm infants have reduced bone mineralization is inversely correlated with gestational age and birth weight. Therefore, the less drastic change in our cohort is not surprising as it consisted of a cohort of premature infants born in the last trimester that were not VLBW and will therefore have less change in mineralization over time. Future studies may look more closely at the impact that kinesthetic movement and massage have independent of each other on bone. However, as kinesthetic movement and massage are often congruent, distinguishing their individual impact is of limited importance.

Together this evidence lends support to the idea that TKS positively affects postnatal bone growth and would provide a low risk, non-invasive means to enhance bone mineralization during early development. We targeted a cohort of preterm infants born at 29 to 32 weeks PMA without evidence of intrauterine growth restriction to address the impact of TKS on appropriately grown, healthy preterm infants. While this cohort comprises most preterm births and minimizes confounding variables, sample size was small and attrition due to infant discharge limited reporting to a two week duration. Given that altered bone mineralization is still detected at childhood [7], follow-up studies to address long-term impact of TKS are warranted. Finally, while we speculate that TKS results in a modification in bone turnover in favor of bone accretion, it is important to recognize that tSOS assesses a specific area of bone while biomarkers in urine are surrogate markers for total body bone turnover. Most bone biomarkers, including U-MidOC, are known to have circadian variation. While every effort was taken to minimize uncontrolled differences between treatment groups, these factors may have influenced results. Recognizing these limitations, evidence suggests that TKS improves bone mineralization.

Disclosure

All authors state that they have no conflicts of interest.

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