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Original Article

Hylonome

Tactile and Kinesthetic Stimulation (TKS) intervention improves outcomes in weanling rat bone in a neonatal stress model

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Abstract

Objectives: Preterm infants are born with low bone mineral. Neonatal stress further impedes bone mineralization. Clinical evidence suggests that tactile and kinesthetic stimulation (TKS) improves bone phenotype and decreases stress response. Clinical and translational studies indicate the IGF-1 axis, responsible for postnatal growth and bone mineralization, is a key player. We hypothesized that TKS would attenuate the negative impact of neonatal stress on bone phenotype and the IGF-1 axis in weanling rats. **Methods:** Neonatal stress (STRESS) or TKS (STRESS + 10min TKS) was administered from D6 to D10. Control animals received standard care. Tissue was harvested on D21. Dual energy x-ray absorptiometry (DXA) and bone morphometry were performed and serum osteocalcin, type I procollagen N-terminal propeptide (PINP), tartrate-resistant acid phosphatase (TRAP), and bone and liver mRNA levels of IGF-1, IGF-1 receptor (IGF-1R), and growth hormone receptor (GHR) were measured. **Results:** Neonatal stress increased bone mineral content (BMC), area (BA), growth plate width, liver IGF-1 mRNA, and serum IGF-1. TKS maintained areal bone mineral density (aBMD) and bone specific IGF-1 and IGF-1R mRNA while STRESS decreased compared to controls. **Conclusions:** Neonatal stress results in apparent accelerated growth response. TKS differed from STRESS with improved tibia aBMD and increased bone specific IGF-1 mRNA.

Keywords: Tactile-Kinesthetic Stimulation, Bone Mineral Density, Bone Mineral Content, IGF-1

Introduction

Preterm birth (30-37 wk GA) results in low bone mineral that persists in children and young adults. When children (3-5 yrs), infants born prematurely exhibit smaller, less mineralized bone as compared to term children and have a higher fracture rate than full term peers[1-3]. Additionally, premature infants are exposed to many necessary caregiving procedures that activate stress systems[4]. Stress hormones increase visceral adiposity, decrease lean mass, and suppress osteoblastic activity responsible for bone formation[5,6].

Infant massage that includes both tactile and kinesthetic stimulation (TKS) attenuates the negative impact of premature birth on bone. Clinical studies demonstrate that TKS increases bone growth, bone strength, and mineral acquisition[7-9]. Further, TKS in the form of infant massage improves ANS response and decreases hormonal markers for stress such as cortisol and epinephrine[10-15].

Studies using translational animal models of neonatal stress detected lasting negative impacts on bone and body composition that were ameliorated by TKS. Neonatal stress in these models increased signs of metabolic derangement with elevated fat mass and hyperinsulinemia in male rats. Neonatal stress also altered bone phenotype and impaired bone mineralization in adolescent rats[16-18]. TKS in these and other models resulted in persistent changes that benefited bone mineralization. TKS prevented hyperinsulinemia in males, increased lean mass, elevated bone mineral content (BMC), and improved markers of bone turn over and microstructure in rodents[16,19].

Insulin-like growth factor (IGF-1) is an important mediator of the effect of growth hormone on bone growth and functions via the AKT signaling pathway to stimulate cell growth and

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proliferation. Elevated expression of IGF-1 and related pathways may explain improved BMC[20]. Clinically, higher levels of circulating IGF-1 and increased weight gain have been detected in preterm infants who received TKS compared to preterm controls[21-23]. Haley et. al. reported that adolescent rats stressed as neonates expressed higher IGF-1 mRNA in tibia tissue when TKS was given during the stress[16]. Elevated bone specific IGF-1 was associated with improved BMC and tibia growth plate width. IGF-1 has been reputed to stimulate growth plate chondrocyte proliferation and hypertrophy[24]. This suggests a persistent impact of neonatal TKS on the IGF-1 axis into adolescents.

The early impact of TKS on bone and the IGF-1 axis has not been evaluated. We investigated the ability of TKS intervention to attenuate the negative impact of neonatal stress on bone in weanling rat pups. Our translational research utilized a rat model to investigate the mechanisms by which TKS, during periods of neonatal stress, affected bone development and IGF-1 levels. We combined multiple stressors (e.g. maternal separation, needle puncture, hypoxia/hyperoxia challenges), which are similar to those encountered by infants in an intensive care environment, to examine whether TKS increased IGF-1 levels and improved bone phenotype. While neonatal rats (postnatal days 5-10) were not 'premature', they allowed an evaluation of the impact of similar stressors in the early postnatal period. Further, the maturity of several organ systems in neonatal rats at birth, particularly brain development, has been estimated to be similar to human infants in the third trimester[25-27]. Measures included bone mineralization, circulating markers of bone formation and resorption, and IGF-1 in serum and bone specific mRNA levels. Little is known about the potential of TKS during the neonatal period to improve outcomes associated with neonatal stress.

Methods

Animals

Timed pregnant Sprague-Dawley dams fed standard rat chow were allowed to deliver spontaneously at term (E21). Litters for each dam were derived from multiple dams (5 in total) to control for genetic effects and to account for different litter sizes. Final litters for each dam contained pups from each of the 5 dams and were composed of 10 pups (5 M, 5 F). Litters were randomly assigned to one of 3 groups: naïve control (CTL), neonatal stress control (STRESS), and neonatal stress with tactile and kinesthetic stimulation (TKS). Serum was collected from two litters of STRESS and TKS, however, bodies from a subgroup of each litter were needed in experiments that precluded them from inclusion in analyses of tissues. Pups were cross fostered daily from day 5 to day 20. Litters were cross-fostered across dams to control for differences in maternal care until weaning at day 20. Weaned rats were removed from dam and fasted for 24 hours before harvest on day 21 (D21). All procedures were approved by the University of Utah Animal Care Committee IUCAC and are in accordance with the American Physiological Society's guiding principles.

Interventions: naïve control (CTL), neonatal stress (STRESS) and neonatal stress with tactile and kinesthetic stimulation (TKS)

Interventions were performed from D6 to D10 of neonatal life. Naïve control (CTL) animals did not undergo stress treatment but otherwise received the same cross-fostering, maintenance, housing, and care as the rats in other groups. Neonatal stress intervention, which was given to both STRESS and TKS, was based on well-established models of neonatal stress[17,18,28]. Neonatal stress intervention is meant to mimic stressors encountered in the neonatal intensive care unit (NICU) such as painful procedures (IVs, blood draws), hypoxic and hyperoxic events, and maternal separation. The stress protocol was performed as previously described[16,17]. In brief, the pup received a needle puncture, a hypoxic and a hyperoxic challenge, with maternal separation lasting for a total of 60 minutes. Hypoxia and hyperoxia treatments were carefully tracked with an oxygen sensor inside the chamber to ensure similar treatments. Hypoxia was achieved by streaming humidified gas mixture (100% N_2) for 8 min into a 22 liter closed container in which the rat pups were placed. Immediately following the hypoxic challenge, hyperoxic conditions were achieved by flushing the chamber with 100% O_2 for 4 minutes. The temperature was maintained at 37°C. The TKS rat pups underwent the same stress treatment pattern except that during the final maternal separation period they were removed individually and provided 10 minutes of tactile stimulation with a soft camel hair brush to the ventral and dorsal body and kinesthetic movement that involves a range of motion movement to the fore and hind limbs as previously described[16,17].

Body mass and bone phenotype

Body mass was measured on an electronic scale (APX-203, Denver Instrument) to the nearest 0.01 g on D6 and D21 of life. Dual energy x-ray absorptiometry, DXA (pDEXA, Norland, Fort Atkinson, WI), calibrated for small-animal research, determined bone area (cm^2), bone mineral content (mg), and areal bone mineral density (mg/cm^2) on D21 rats prior to harvest (n=10 CTL, n=20 STRESS, n=20 TKS). DXA measurements assessed the whole body and were performed while lightly anesthetized with isoflurane. The daily coefficient of variation (CV) was 0.6% for the DXA manufacturer phantom and the CV for repeated scans and standards was <1.0%.

Growth plate morphometry and tibia length

The right tibia was harvested on Day 21 and preserved in 10% buffered formalin, dehydrated, and embedded in methyl methacrylate for histomorphometry. The femur was sectioned proximal to the femoral condyles in order to conserve the proximal tibia growth plate. Using a caliper, tibia length was measured and recorded (n=10/TX). Frontal sections of the proximal tibia were cut at 7 μm using a microtome equipped with tungsten carbide steel blades. The sections were mounted on Superfrost slides and stained with Toluidine Blue prior to analysis of the growth plate. The widths of the growth plate were measured in 5 equidistant regions in the central 1/3 of the growth plate using

an image analyzer (Bioquant Nova Prime). An average of the 5 measurements for each animal was calculated (n=10/TX).

Serum bone turnover markers

Osteocalcin, a specific product of osteoblasts, was measured by ELISA (Biomedical Technologies, MA) using a monoclonal antibody directed against the N-terminal region of both carboxylated (Gla-OC) and undercarboxylated (Glu-OC) rat osteocalcin (ng/mL). Serum N-terminal propeptide of type I procollagen (PINP; Immunodiagnostic Systems, AZ) was determined by quantitative EIA. PINP (ng/mL) is indicative of collagen synthesis and therefore systemic bone formation activity. Osteoclast-derived tartrate resistant acid phosphatase form 5b (TRAP) was determined with a solid phase immunofixed enzyme assay (Immunodiagnostic Systems AZ). TRAP (U/L) is secreted specifically by osteoclasts and is indicative of systemic osteoclastic activity. Blood collected at harvest was centrifuged at 2,987g for 10 minutes and serum was collected and stored at -20°C until analysis. All samples were run in duplicate and per manufacturer instructions (n=10 CTL, n=20 STRESS, n=20 TKS).

Serum IGF-1, and IGFBP-3

Serum IGF-1 (R&D Systems, MN) and serum IGFBP-3 (Mediagnost, Germany) were measured in duplicate by ELISA assay (ng/mL) as per manufacturer guidelines (n=10 CTL, n=20 STRESS, n=20 TKS).

Real-time reverse transcriptase PCR

The abundance of liver IGF-1 and IGF-1 receptor, and bone-specific IGF-1, IGF-1 receptor, and growth hormone receptor (GHR) messenger RNA was evaluated with real-time reverse transcriptase PCR. At harvest, the left tibia was removed, conserving the tibial growth plate, all muscle was removed, and bone was immediately placed in liquid nitrogen. The left tibia was crushed under liquid nitrogen using a Certiprep 6750 Freezer Mill until a fine powder was produced. Total mRNA was extracted from the crushed tibia using a RNeasy Lipid Tissue RNA purification kit (Qiagen, MD) according to manufacturer instruction and stored at -80°C (n=10/TX). Total RNA was quantified using a NanoDrop 3300 Fluorospectrometer (Thermo Scientific, DE) and visualized by gel electrophoresis. Synthesis of cDNA was done with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) from 1µg of total RNA. The following Assay-on-demand primer/probe sets were used: IGF-1 – Rn00710306_m1; IGF-1 receptor - Rn01477918_m1; GHR – Rn00567298_m1 (Applied Biosystems, CA).

Quantification of mRNA abundance was determined using the comparative Ct method[29] with GAPDH as an internal control (GAPDH primer and probe sequences; Forward: CAAGATGGTGAAGGTCGGTGT; Reverse: CAAGAGAAGGCAGCCCTGGT; Probe: GCGTCCGATACGGCCAAATCCG). All real-time PCR amplification, data acquisition, and analysis were done using the 7900 HT Real-time PCR system and SDS Enterprise Software (Applied Biosystems, CA) using a 384-Well Optical Reaction Plate (Applied Biosystems, CA). Taqman Uni-

	Males	Females
Body Mass (g)		
CTL	51.9±1	49.4±3
STRESS	55.2±3*	51.3±3
TKS	52.8±2	51.0±2
Tibia Length (cm)		
CTL	2.0±0.08	1.9±0.08
STRESS	2.0±0.08	1.9±0.13
TKS	2.0±0.05	2.0±0.10
Bone mineral content, BMC (g)		
CTL	0.31±0.02	0.30±0.05
STRESS	0.36±0.02*	0.34±0.05*
TKS	0.34±0.04	0.37±0.04*
Bone Area, BA (cm²)		
CTL	6.7±0.5	7.0±1.3
STRESS	8.7±0.4*	8.5±0.7*
TKS	8.2±0.6*	8.6±0.9*
Areal bone mineral density, aBMD (g/cm²)		
CTL	0.046±0.002	0.047±0.003
STRESS	0.044±0.002^	0.044±0.003^
TKS	0.045±0.003	0.045±0.002

*Data are expressed as mean±SD: Naïve control (CTL); Neonatal stress (STRESS); Neonatal stress with tactile kinesthetic stimulation (TKS). All factors were analyzed with body mass as a covariate. Where significant (p≤0.05) * denotes >CTL and ^ denotes <CTL.*

Table 1. Body Mass, Tibia Length, and Bone Measures with Dual X-Ray Absorptiometry (DXA).

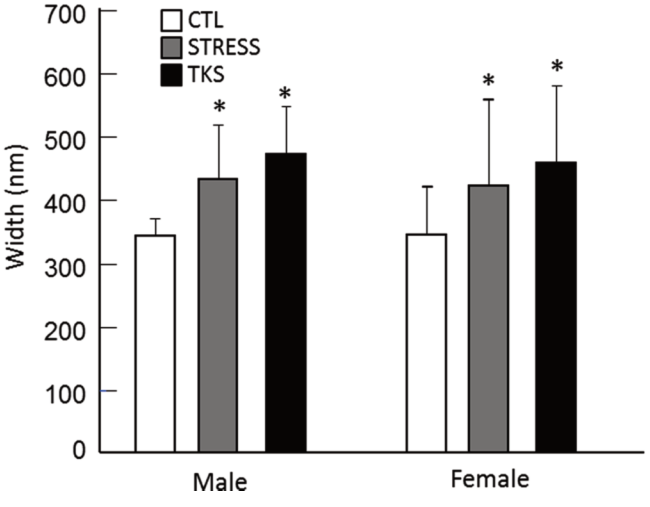

Figure 1. Epiphyseal plate morphometry plotted mean width ± SD for naïve control (CTL), neonatal stress (STRESS), and neonatal stress with tactile kinesthetic stimulation (TKS). Where significant (p≤0.05) the * signifies >CTL.

	Total Osteocalcin, OC (ng/mL)	Undercarboxylated Osteocalcin, unOC (ng/mL)	Propeptide of type I collagen, PINP (ng/mL)	Tartrate resistant acid phosphatase, TRAP (U/L)	Insulin-like Growth Factor-1, IGF-1 (ng/mL)	IGF Binding Protein 3, IGFBP-3 (ng/mL)
Male						
CTL	162±70	36.4±9	34.2±15	4.6±1	249±48	0.72±0.2
STRESS	147±73	48.5±6*	26.6±7	8.7±3*	301±51	0.62±0.3
TKS	130±42	47.2±11	25.4±16	7.5±2*	308±48*	0.60±0.2
Female						
CTL	131±86	45.4±14	33.5±13	5.6±1	204±48	0.76±0.2
STRESS	155±61	38.4±9	32.0±14	8.2±3*	345±33*	0.63±0.1
TKS	193±75	44.1±7	31.7±19	7.2±2*	297±57*	0.60±0.2

*Data are expressed as mean ± SD: Naïve control (CTL); Neonatal stress (STRESS); Neonatal stress with tactile kinesthetic stimulation (TKS). Where significant (p≤0.05) * denotes > CTL.*

Table 2. Serum bone turnover markers, IGF-1, and IGFBP3 levels.

versal PCR Mastermix (Applied Biosystems, CA) was used in a 5 μL reaction, performed in quadruplicate. Cycle parameters were: 50°C x 2 min, 95°C x 10 min, followed by 40 cycles of 95°C x 15 sec and 60°C x 60 sec.

Statistical analysis

Bone densitometry, serum levels, mRNA expression, tibia length, and growth plate morphometry results were analyzed using a two-way analysis of variance (ANOVA) with treatment and sex as the independent variables. When significant ($P<0.05$), a post-hoc Fisher's LSD for pair-wise comparisons was conducted. Because there were differences in body mass, an ANCOVA was used in DXA analyses to compare treatments using body mass (g) as a covariate and treatment and sex as the independent factors. Systat 10 (SPSS Inc., Chicago, IL) was used for analysis.

Results

Body mass and tibia length

Body mass taken at day six (D6) before interventions started was not statistically different for all treatment groups (mean±SD; CTL 14.51±0.8 g; STRESS 15.2±0.9 g; TKS 15.3±1.1 g). Body mass taken at weaning (D-21) was six percent heavier in STRESS rats than controls (Table 1; Tx effect p<0.05). This difference was primarily due to an increase in STRESS male weight (Fisher's LSD p<0.05). Females weighed six percent less than males (Sex effect p<0.001). Tibia length did not differ among treatment groups or between sexes (Table 1).

Epiphyseal growth plate morphometry

The epiphyseal growth plate width was more than 30% wider in treatment groups that underwent stress (STRESS and TKS) compared to naïve control (Figure 1. Tx effect p<0.001; Fisher's LSD p<0.05). There were no sex or interaction effects.

Bone densitometry by Dual X-Ray Absorptiometry (DXA)

Neonatal stress in our model resulted in approximately 13% greater BMC and 22% greater Bone Area (Tx effect p≤0.001; Fisher's LSD p<0.05) in weanling rats with the exception of TKS males exhibiting only a trend toward higher BMC (p=0.09). Areal bone mineral density (aBMD) is calculated based on the mineral mass (BMC) per unit of bone area (BA) as detected by DXA. Because of differences in BMC and BA, STRESS animals had 7% less aBMD than control, while TKS animals were statistically similar to control (Table 1. Tx effect p≤0.01; Fisher's LSD p<0.05)

Serum bone turnover markers

There were no differences in total osteocalcin levels among treatment groups or between sexes. Undercarboxylated osteocalcin was higher in STRESS males compared to CTL males (Fisher's LSD p<0.05). There were no differences among females resulting in an interaction effect (p<0.05). PINP was similar across all groups and between sexes. Bone resorption marker, TRAP, was elevated in STRESS by almost two-fold in males and by 50% in females (female p=0.002; male p=0.003) and in TKS by approximately 50% (female p=0.03; male p=0.05) compared to CTL (Tx effect p<0.001). None of the serum bone turnover markers differed between sexes (Table 2).

Serum IGF-1 and IGFBP-3

Serum Insulin-like Growth Factor-1 (IGF-1) was elevated in both female stress groups (STRESS and TKS) compared to CTL (Tx effect p<0.001; Fisher's LSD p<0.002). Serum IGF-1 in males was elevated in TKS compared to CTL (Fisher's LSD p<0.05) with a trend in STRESS (p=0.07). There were no differences among treatment groups for serum IGFBP-3 (Table 2).

Messenger RNA levels of IGF-1, IGF-1 Receptor, and GH Receptor by Real-time RT PCR

Liver IGF-1 mRNA expression levels were nearly 2.5 times higher (p<0.001) in both neonatal stress groups (STRESS and

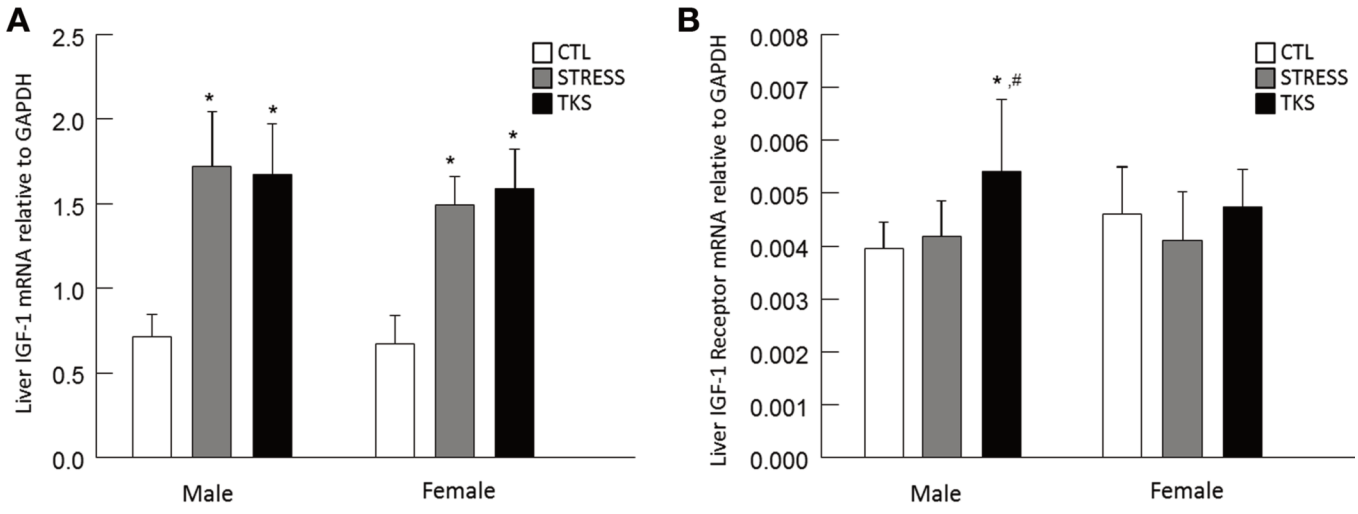

Figure 2. Liver mRNA. **A.** Liver IGF-1 mRNA expression relative to GAPDH. **B.** Liver IGF-1 Receptor mRNA expression relative to GAPDH. Plotted are means ± SD for naïve control (CTL), neonatal stress (STRESS), and neonatal stress with tactile kinesthetic stimulation (TKS). Where significant ($p \leq 0.05$) the * signifies >CTL and # signifies >STRESS.

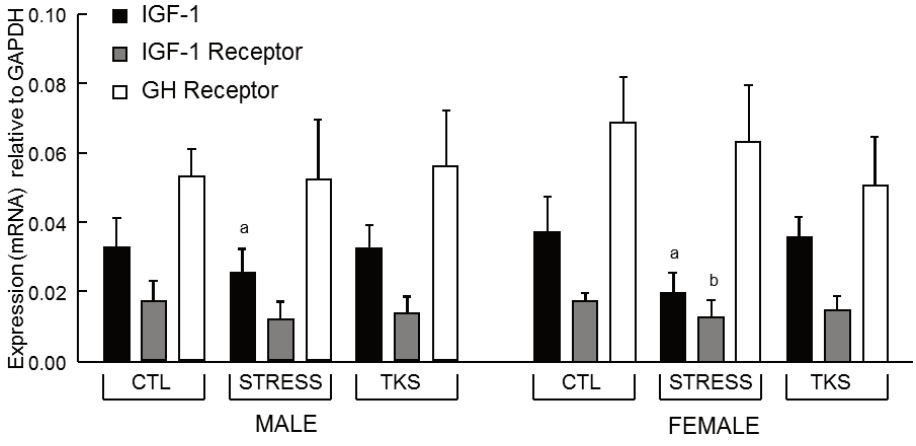

Figure 3. Bone specific mRNA expression relative to GAPDH for IGF-1 (black), IGF-1 Receptor (gray), and GH Receptor (white). Plotted are means ± SD for each mRNA level separated by naïve control (CTL), neonatal stress (STRESS), and neonatal stress with tactile kinesthetic stimulation (TKS). Where significant ($p \leq 0.05$) the 'a' signifies < both CTL and TKS. The 'b' signifies < CTL.

TKS) compared to control liver mRNA (Figure 2A). Liver IGF-1 receptor mRNA analysis resulted in significant treatment ($p=0.002$) and interaction ($p<0.05$) effects that were explained by higher IGF-1 receptor levels in TKS males compared to both CTL and STRESS (Fisher's LSD $p<0.002$). Females, however, did not differ among treatments (Figure 2B).

Bone specific IGF-1 mRNA expression levels were decreased in STRESS compared to CTL and TKS in both males and females (Tx effect $p<0.001$; Fisher's LSD $p<0.05$ for males and $p<0.001$ for females). Furthermore, bone specific IGF-1 receptor mRNA levels were decreased in female STRESS compared to CTL, but not TKS (Tx effect $p<0.05$; Fisher's LSD $p<0.05$) with a trend in males ($p=0.06$). No significant differences were detected in GH receptor mRNA levels (Figure 3). Further, no differences were detected in mRNA expression levels between males and females for IGF-1, IGF-1 receptor, or GH receptor.

Discussion

Premature infants are at increased risk for delayed growth and bone mineralization especially after prolonged hospitalization in an intensive care unit[30]. Hospitalized infants develop physiological instability during necessary caregiving and have

heightened stress hormone exposure[4,31-33]. Chronic exposure to stress hormones is associated with reduced growth as a result of growth hormone suppression and development of IGF-1 resistance[5]. This can be seen in children with anxiety disorders who have slower growth and increased risk of osteoporosis later in life[34]. Despite improved neonatal care, early birth and postnatal stressors result in sub-optimal bone mineralization (osteopenia of prematurity). Tactile and kinesthetic stimulation (TKS) has been advocated as a means to improve bone mineralization in these infants. Both clinical and translational studies support such assertions. We hypothesized that neonatal stress would negatively impact the IGF-1 axis resulting in impaired mineralization in weanling rats. Further we predicted that TKS would ameliorate the negative impact on bone development and IGF-1 levels. We found that neonatal stress (STRESS and TKS) resulted in greater BMC, BA, growth plate width, as well as greater liver IGF-1 mRNA and serum IGF-1 compared to control. TKS maintained areal bone mineral density (aBMD) while STRESS rats had reduced aBMD compared to control. Further, TKS animals maintained bone specific IGF-1 and IGF-1 receptor mRNA while levels were reduced in STRESS compared to controls. In summary, neonatal stress groups appeared to be in a state of accelerated bone growth. The impact of TKS was primarily on bone specific mRNA expression of IGF-1 related genes.

IGF-1 is a key player in postnatal bone area expansion and mineral deposition. IGF-1 is released into circulation via hepatic production and is also produced locally in muscle and bone to act in a paracrine manner. Bone specific IGF-1 is requisite for normal skeletal development and mineral acquisition[35]. IGF-1 increases bone matrix formation and osteoblast maturation[24]. Glucocorticoids released in response to stress suppress IGF-1 transcription in osteoblasts, the cells responsible for bone formation[36-38]. Congruent with our current finding, we previously detected elevated IGF-1 and IGF-1 receptor mRNA levels in tibia bone from adolescent rats treated with TKS during neonatal stress[16]. The impact of TKS on bone specific IGF-1 mRNA therefore appears to start early and persist. Importantly, in adolescent TKS rats, bone specific increases in IGF-1 mRNA were associated with increased BMC and aBMD[16]. Osteoblast IGF-1 axis signaling is an essential determinate in the level of bone mineralization. The persistent elevation in IGF-1 mRNA suggests a long-term benefit of neonatal TKS presented during neonatal stress. Higher mRNA does not necessarily confirm elevation in protein expression and translational up regulation would need to be confirmed. We speculate, however, that this difference between TKS and STRESS in IGF-1 mRNA may explain the improved aBMD at weaning and may lead to the improvements previously detected in adolescent TKS rat bone. While circulating IGF-1 has a clear and important role in growth, local production of IGF-1 helps mediate the skeletal growth promoting action of growth hormone independently of circulating IGF-1[39,40]. Investigation of the role of autocrine/paracrine IGF-1 in TKS driven growth and bone mineralization is warranted.

Neonatal stress is often followed by a period of catch-up growth. For example, children who are dexamethasone-exposed or experience intrauterine growth restriction later experience accelerated growth. Indeed, accelerated weight gain out to D21 immediately following neonatal stress has been detected in both STRESS and TKS rats compared to control in this model[17]. Under normal conditions, postnatal longitudinal bone growth is rapid then slows and later ceases with age. During stress, glucocorticoids slow the process of growth plate senescence to conserve the growing capacity of the growth plate until growth inhibiting conditions resolve[41]. Evidence suggests that the phenomenon of catch-up growth is, in part, due to delayed growth plate senescence[42]. The rate of growth at the growth plate is controlled by several endocrine signals, including IGF-1. However, the molecular mechanism that slows growth is not well understood and senescence appears to occur due to growth itself and proliferative activity of stem-like cells at the growth plate[42]. Catch-up growth has been detected following transient glucocorticoid exposure and associated with an elevation in serum IGF-1 with growth plate chondrocyte proliferation[41]. For example, postnatal rat metatarsal bones in culture exposed to dexamethasone for 7 or 12 days experienced decreased chondrocyte proliferation and differentiation during exposure[43]. After exposure ceased, catch-up growth was characterized by increased chondrocyte proliferation. Addition of IGF-1 increased the hypertrophic zone of the growth plate 3-fold, reversing the growth inhibition of dexamethasone on the growth plate[44]. We speculate that neonatal stress groups have delayed growth during neonatal stress and that greater growth plate width, elevation in serum IGF-1, and increased markers of bone turnover in stress groups may be indicative of catch-up growth following the period of neonatal stress. Rapid growth may also contribute to the greater bone area and bone mineral content detected in stress groups resulting in altered aBMD measures compared to control animals. However, despite no differences in tibia length, STRESS rats appeared to suffer long-term negative impacts to bone that TKS ameliorated. A study of STRESS and TKS adolescent rats found that STRESS BMC, aBMD, growth plate width, and markers of bone formation were reduced in later life compared to control and TKS[16]. Circulating IGF-1 has the capacity to compensate for decreased bone specific IGF-1, however this is primarily seen postpubertally, not postnatally in transgenic models[35,45]. Further, osteoblast specific IGF-1 in transgenic models increased trabecular bone volume whereas circulating IGF-1 altered cortical bone volume[46-48]. Future studies need to perform mechanical testing of bone strength and evaluate the microarchitecture of trabecular and cortical bone as these are limitations to the current study. Further, bone and growth parameters need to be measured immediately during and following neonatal stress to confirm the assumption that signs of accelerated growth were preceded by delayed tibial bone growth during stress. The inclusion of a TKS intervention in the absence of a stress model would also expand our understanding of the impact TKS has on IGF-1 axis under normal conditions.

Modeling and remodeling continually shape bone with formation via osteoblasts and resorption via osteoclasts. In grow-

ing skeletons, modeling usually predominates to form new bone and enable linear growth and bone area expansion. Serum markers have suggested that TKS may increase formation and decrease resorption compared to STRESS. Previously, TKS intervention resulted in elevated serum osteocalcin and reduced serum TRAP in adolescent rats[16]. Further, animal models of tactile stimulation alone found decreased eroded surface and decreased serum TRAP coupled with improved BMC and aBMD, suggesting decreased resorption[19]. Interestingly, glucocorticoids prolong the survival of osteoclasts responsible for resorption[49-52]. Weanling neonatal stressed rats had higher TRAP than control rats. Maintenance of osteoclast numbers and activity would result in loss of bone density. Lower bone density compared to control was only seen in STRESS weanling rats. The magnitude of difference in TRAP levels was much higher in STRESS compared to TKS suggesting potentially higher resorption activity. Further, elevation in undercarboxylated osteocalcin, associated with greater resorption, was detected in STRESS males but not TKS. Clinically, preterm infants receiving TKS have not shown signs of reduced pyridinium crosslinks, a biochemical marker of resorption, but have consistently shown increased tibia speed of sound or bone strength indicating greater bone mineralization[7,8,53]. Taken together, TKS may result in a positive balance between bone formation and resorption that improves long-term outcome to bone phenotype.

Catch-up growth is associated with increased risk for metabolic disturbances. Early development is a critical period during which stress can result in perturbations to growth quality and metabolic consequences[54]. Circulating IGF-1 is structurally similar to insulin and results in insulin-like effects (i.e. increased adipogensis, glucose uptake, and inhibition of gluconeogenesis)[40]. Further, bone remodeling impacts glucose, lipid, and energy metabolism. Insulin is a key molecular player in the connection of bone remodeling and energy metabolism. Insulin indirectly enhances bone resorption by osteoclasts and the release of the metabolicly active undercarboxylated form of osteocalcin[55]. Interestingly, measures of body composition in neonatal stressed rats at weaning following accelerated growth detected greater total body fat mass, particularly abdominal subcutaneous fat and, in males, abdominal visceral fat compared to control[17]. Further, hyperinsulinemia is detected in STRESS males in both weanling and adult rats[17,56]. IGF-1 and insulin have lipogenic properties and have been found to be associated with substantial gain in fat mass[57,58]. The elevation in serum IGF-1 detected here may reveal a major factor by which neonatal stress accelerated growth and promoted fat deposition. In preterm infants, studies found IGF-1 to be correlated with postnatal growth[59]. Preterm infants also had greater fat mass, particularly visceral fat, at 40 weeks corrected age compared to term infants[60-63]. Interestingly, TKS interventions for preterm infants, such as infant massage, have conflicting results with some detecting elevated serum IGF-1 and others no change[21,64]. There are reports that infant massage improved growth quality and reduced fat mass in male preterm infants[64]. Quality of body composition is an important determinate in the etiology of metabolic syndrome and increased fat mass resulting from neonatal stress may predispose preterm infants to long-term negative metabolic impacts.

In summary, treatment of neonatal stressed rat pups with TKS helped maintain aBMD and bone specific mRNA of IGF-1 to control levels. Neonatal stress in both groups appeared to accelerate bone growth. Both TKS and STRESS had wider epiphyseal growth plate width, greater markers of bone turnover, and bones with greater area and mineral content compared to control. The elevation in serum IGF-1 in neonatal stress groups likely contributed to accelerated growth and to altered bone phenotype. In light of previous studies of neonatal stress and TKS, the improvements to BMC and bone quality fall off for STRESS groups but are maintained in TKS in the long-term. This is consistent with many studies that suggest catch-up growth may result in long-term increased propensity for adult disease. However, the current study suggests a prolonged, positive impact of TKS on skeletal health that is primarily due to differences in bone specific expression of IGF-1 mRNA. Future research will need to clarify whether TKS alters IGF-1 expression by means of stress reduction or mechanical stimulation or both. The persistent change in bone specific IGF-1 mRNA suggests there may be alteration in programming of the IGF-1 gene. Altered epigenetic characteristics of IGF-1 gene have previously been demonstrated in a rat model for intrauterine growth restriction (IUGR)[65]. The current study encourages future investigation into epigenetic programming of IGF-1 by TKS in bone, particularly in the case of neonatal stress. The Developmental Origins of Disease Hypothesis emphasizes that stress during 'critical periods' of development results in negative life-long consequences that include impaired skeletal health. TKS may offer a noninvasive means to ameliorate the negative impact of neonatal stress on skeletal health in preterm infants.

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