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Original Article

Hylonome

Mechanical-Tactile Stimulation (MTS) intervention in a neonatal stress model improves long-term outcomes on bone

S. Haley[1], S. O'Grady[1], K. Gulliver[1], B. Bowman[2], R. Baldassarre[1], S. Miller[2], R.H. Lane[3], L.J. Moyer-Mileur[1]

[1]Center for Pediatric Nutrition Research; [2]Department of Radiology; [3]Division of Neonatology, University of Utah, Salt Lake City, Utah

Abstract

Objectives: Neonatal stress impairs postnatal bone mineralization. Evidence suggests that mechanical tactile stimulation (MTS) in early life decreases stress hormones and improves bone mineralization. Insulin-like growth factor (IGF1) is impacted by stress and essential to bone development. We hypothesized that MTS administered during neonatal stress would improve bone phenotype in later life. We also predicted an increase in bone specific mRNA expression of IGF1 related pathways. **Methods:** Neonatal stress (STRESS) and MTS (STRESS+10 min of MTS) were given from D6 to D10 of rat life and tissue was harvested on D60 of life. Dual energy x-ray absorptiometry (DXA), bone morphometry, serum osteocalcin, type I procollagen N-terminal propeptide (PINP), tartrate-resistant acid phosphatase (TRAP), and bone and liver mRNA levels of IGF1, IGF1 receptor (IGF1R), and growth hormone receptor (GHR) were measured. **Results:** Stress resulted in reduced bone area and bone mineral content (BMC) compared to naïve control (CTL). MTS intervention increased BMC and tibial growth plate width compared to STRESS. MTS also resulted in higher osteocalcin, and, in males, lower TRAP ($p<0.05$). MTS resulted in three-fold, two-fold, and six-fold higher bone specific IGF1, IGF1R, and GHR, respectively ($p\leq0.001$) compared to STRESS. **Conclusions:** MTS in early postnatal life improves long-term bone mineralization. IGF1 and related pathways may explain improved BMC.

Keywords: Mechanical-tactile Stimulation, Bone Mineral Density, Bone Mineral Content, Insulin-like Growth Factor (IGF-1)

Stress during 'critical periods' of development negatively impacts bone growth with life-long consequences to bone mineralization and strength[1,2]. Infants born prematurely experience repeated postnatal stressors such as maternal separation, painful procedures, and hypoxic/hyperoxic events during hospitalization and additionally begin life with sub-optimal bone mineralization (osteopenia)[3]. Despite major advances in neonatal care, postnatal growth and bone development of preterm infants does not increase as it does *in utero*[3]. This results in long-term negative impacts. Children (3-5 yrs) born mildly premature, 3-10 weeks early (30-37 wk GA), exhibit smaller bones and lower bone mineral content compared to term children[4]. Additional long-term effects of delayed bone mineralization include shorter stature and a higher rate of fracture than full term peers[5-7]. Evidence also suggests a resultant reduction in peak bone mass and increased risk of impaired bone health into adulthood.

Clinical and animal model studies suggest that manual interventions may attenuate the negative impact of premature birth on bone. Clinically, kinesthetic movement of extremities in preterm infants increases linear growth, bone mineralization, and decreases bone resorption[8-12]. Evidence suggests this may be the result of reduced stress hormones. Tactile stimulation in the context of infant massage can decrease hormonal markers for stress that negatively impact bone such as cortisol and epinephrine[13-18]. Animal models offer similar findings. Rodent neonatal stress models, such as maternal separation, pain/fear, or undernutrition, have multiple negative impacts on physical growth. Neonatal stress in these models slows growth, increases adiposity and leptin, and increases the risk of adult morbidities[19-27]. In response to massage-like stroking referred to as tactile stimulation, neural and endocrine stress markers

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Corresponding author: Shannon Haley, PhD, LMT, Center for Pediatric Nutrition Research, Department of Pediatrics, University of Utah, 295 Chipeta Way, Salt Lake City, UT 84108
E-mail: shannon.haley@hsc.utah.edu

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are reduced. For example, autonomic nervous system tone is improved[28], rodents exhibit increased weight gain[29], and increased release of gastric hormones and growth factors[28,29]. One study found that neonatal tactile stimulation in a non-stress rodent model increases bone strength, improves bone architecture, and decreases bone resorption[30]. Little is known about the effect of mechanical/tactile stimulation (MTS) on bone in the context of a stress model. MTS is explored as a viable intervention to improve bone growth and development in a rodent model of neonatal stress.

It has been postulated that the somatotrophic axis is a participating mechanism by which MTS improves growth. Postnatal linear growth and bone mineralization are regulated by the somatotrophic axis which includes growth hormone (GH) and insulin-like growth factor-1 (IGF-1)[31,32]. Circulating as well as bone-specific expression of IGF-1 play an essential role in bone development[32]. In skeletal tissue, IGF-1 is primarily synthesized by osteoblasts and has an important role in maintenance of bone mass[31]. IGF-1 enhances differentiation and mitogenic activity of osteoblasts and ultimately increases bone matrix while also decreasing its degradation[31,33]. IGF-1 is positively impacted by both mechanical stimulus and reduction in chronic exposure to stress hormones. Glucocorticoids negatively impact osteoblast activity directly and cause additional inhibition of bone formation by transcriptional down-regulation of IGF-1 in osteoblasts[34]. In clinical studies, preterm infants receiving massage and kinesthetic movement have elevated circulating IGF-1 levels[35]. Positive effects of MTS on weight gain, body composition, and bone mineral acquisition may involve alterations to the somatotrophic axis.

We hypothesized that MTS intervention in a translational model for neonatal stress would improve bone mineralization and biochemical markers of bone homeostasis. Furthermore, we predicted that the IGF-1 axis is a participating mechanism in improved bone development by MTS. This translational research utilized a rat model of neonatal stress to investigate the mechanisms by which MTS affects long-term bone growth and development. Our model combined multiple stressors (e.g. needle puncture and hypoxia, hyperoxia challenges) that are also encountered by hospitalized, preterm infants. We investigated the ability of MTS intervention to attenuate the long-term negative impact of neonatal stress on bone growth and development assessed by measures of growth, growth plate morphometry, bone mineralization, biomarkers of bone formation and resorption, and circulating as well as bone-specific IGF-1 in adolescent rats.

Methods

Animals

Timed pregnant Sprague-Dawley dams were fed standard rat chow and allowed to deliver spontaneously at term (E21). Litters were culled to 10 pups (5 M, 5 F) and randomly assigned to one of 3 groups: naïve control (CTL), neonatal stress control (STRESS), and neonatal stress with mechanical/tactile stimulation (MTS). A second group of litters were used to augment sample sizes for DXA, tibia length, and growth plate where possible. All pups were cross fostered (D5-D20) to minimize any affect of differing maternal care until weaning at day 21 (D21). Weaned rats received *ad libitum* food and water and were maintained on a 12 hour light cycle until harvest on D60 of life. Animals were fasted for 24 hours before harvest. 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 mechanical/tactile stimulation (MTS)

Interventions were performed from D6 to D10 of neonatal life. Naïve control (CTL) animals were not given any stress treatment and were otherwise treated the same for cross-fostering, maintenance, housing, and care. Neonatal stress intervention, given to both STRESS and MTS, was based on well established models of neonatal stress[26,27,36]. Furthermore, Moyer-Miluer et.al. has previously described MTS treatments[36]. The stress protocol consisted of 60 minutes of maternal separation during which time the pup experiences the following sequence: 1) a needle puncture; 2) a hypoxic challenge achieved with a steady flow of 100% nitrogen gas for 8 minutes in a 22 liter closed chamber followed by room air ($21\%O_2$); 3) a hyperoxic challenge achieved with a steady flow of 100% oxygen gas for 4 minutes in a 22 liter closed chamber; 4) maternal separation for remaining time to 60 minutes. Hypoxia and hyperoxia treatments were carefully tracked with an oxygen sensor inside the chamber to ensure similar treatments. The temperature was maintained at 37°C. The MTS rat pups were provided 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. 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.

Body mass and bone phenotype

Body mass was obtained on an electronic scale (APX-203, Denver Instrument) to the nearest 0.01 g on D6, D21, and D60. Bone area (cm^2), bone mineral content (mg), and apparent bone mineral density (mg/cm^2) were determined by dual energy x-ray absorptiometry, DXA (pDEXA, Norland, Fort Atkinson, WI) on D60 rats prior to harvest (n=14 CTL, 19 STRESS, 18 MTS) with the animal sedated by isoflurane anaesthesia. The DXA, calibrated for small-animal research, assessed bone area (BA, cm^2), bone mineral content (BMC, g), and bone mineral density (areal BMD, g/cm^2) in the area of the truck from sternum to coccyx. The daily coefficient of variation for the manufacturer-supplied phantom was 0.6%. Apparent bone mineral density (aBMD) is calculated based on the mineral mass (BMC) per unit of bone area (BA) as detected by DXA.

Growth plate morphometry and tibia length

Tibia were harvested on Day 60. The femur was sectioned immediately below the femoral condyles in order to conserve the proximal tibial growth plate. Using a caliper, tibia length was measured and recorded (n=12/TX). The right tibia was preserved in 10% buffered formalin, dehydrated, and embedded in methyl methacrylate for histomorphometry. 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 Toludine Blue prior to analysis of the growth plate and hypertrophic zone. The widths of the growth plate and the hypertrophic zone 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=18 CTL, 10 STRESS, 9 MTS).

Serum bone turnover markers

Blood collected at harvest was centrifuged at 2,987g for 10 minutes and serum was collected and stored at -20°C until analysis. 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. 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). Osteoclast-derived tartrate resistant acid phosphatase form 5b (TRACP5b) was determined with a solid phase immunofixed enzyme assay (Immunodiagnostic Systems AZ). TRACP5b (U/L) is secreted specifically by osteoclasts and is indicative of systemic osteoclastic activity. All samples were run in duplicate and per manufacturer instructions (n=10 CTL, 10 STRESS, 9 MTS).

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, 10 STRESS, 9 MTS).

Real-Time reverse transcriptase PCR

Real-time reverse transcriptase PCR was used to evaluate mRNA abundance of liver IGF-1 and IGF-1 receptor, and bone-specific IGF-1, IGF-1 receptor, and growth hormone receptor (GHR). Extraction of mRNA was taken from the left tibia stored at -80°C after harvest. The tibia was trimmed, conserving the tibial growth plate, and all muscle removed. 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 (n=10 CTL, 10 STRESS, 9 MTS). 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[37] 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 7900HT Real-time PCR system and SDS Enterprise Software (Applied Biosystems, CA) using a 384-Well Optical Reaction Plate (Applied Biosystems, CA). Taqman Universal PCR Mastermix (Applied Biosystems, CA) was used in a 5 μL reaction, performed in quadruplicate. Cycle parameters were: 50°Cx2 min, 95°Cx10 min, followed by 40 cycles of 95°Cx15 sec and 60°Cx60 sec.

Statistical analysis

Bone densitometry, serum levels, mRNA expression, tibial 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 independent variables or interaction effects were significant ($p \leq 0.05$), 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

The mean body mass at the beginning of the experiment period (D6) was not statistically different among all treatment groups (naïve CTL 14.51±1.8g; STRESS 15.2±1.6g; MTS 15.3±1.0g). Adolescent rats (D-60) males were approximately 60% larger in body mass compared to females ($ANOVA_{sex\ effect}$ $p \leq 0.001$; Table 1). MTS were larger than both STRESS and CTL adolescent rats ($ANOVA_{treatment\ effect}$ $p \leq 0.001$; Table 1).

Tibial length and growth plate morphometry

Neonatal stress (STRESS) in female rats reduced tibia length compared to naïve CTL (Fisher's LSD p=0.05). MTS attenuated this reduction where MTS and naïve CTL had similar tibia lengths (Table 1). Tibia length was not different among males. Males have a longer tibia than females ($ANOVA_{sex\ effect}$ $p \leq 0.001$). MTS also attenuated a reduction in growth plate width in male rats (Figure 1). Growth plate width was approximately 35% smaller in male STRESS animals compared to both naïve CTL and MTS males (Fisher's LSD p=0.016 and p=0.026, respectively). MTS males have greater hypertrophic width than naïve CTL (Fisher's LSD p=0.006)

	Body Mass (g)	Tibia Length (cm)	Bone Area (cm^2)	Bone Mineral Content (g)	Bone Mineral Density (g/cm^2)
Male					
CTL	369±13	3.64±0.07	34.61±4.2[a,c]	3.45±0.36[a,c]	0.100±0.003
STRESS	388±25	3.62±0.04	21.09±0.9	2.74±0.24	0.130±0.009[b]
MTS	402±20[a,b]	3.62±0.04	20.42±0.6	2.99±0.18[a]	0.140±0.007[a,b]
Female					
CTL	221±6	3.39±0.04[a]	23.81±1.8[a,c]	2.26±0.13[a,c]	0.095±0.004
STRESS	230±19	3.31±0.07	12.78±1.7	1.42±0.23	0.111±0.011[b]
MTS	256±16[a,b]	3.35±0.05	12.71±1.4	1.53±0.12	0.121±0.007[a,b]

Data are expressed as mean±SD: Naïve control (CTL); Neonatal stress control (STRESS); Neonatal stress with mechanical/tactile stimulation (MTS). All factors, except body mass, were analyzed with body mass as a covariate. Where significant (p≤ 0.05) the letters signify [a]: >STRESS; [b]: >CTL; [c]: >MTS.

Table 1. Body mass, tibia length, and bone densitometry by sex and treatment for Sprague-Dawley rats at day 60 of life.

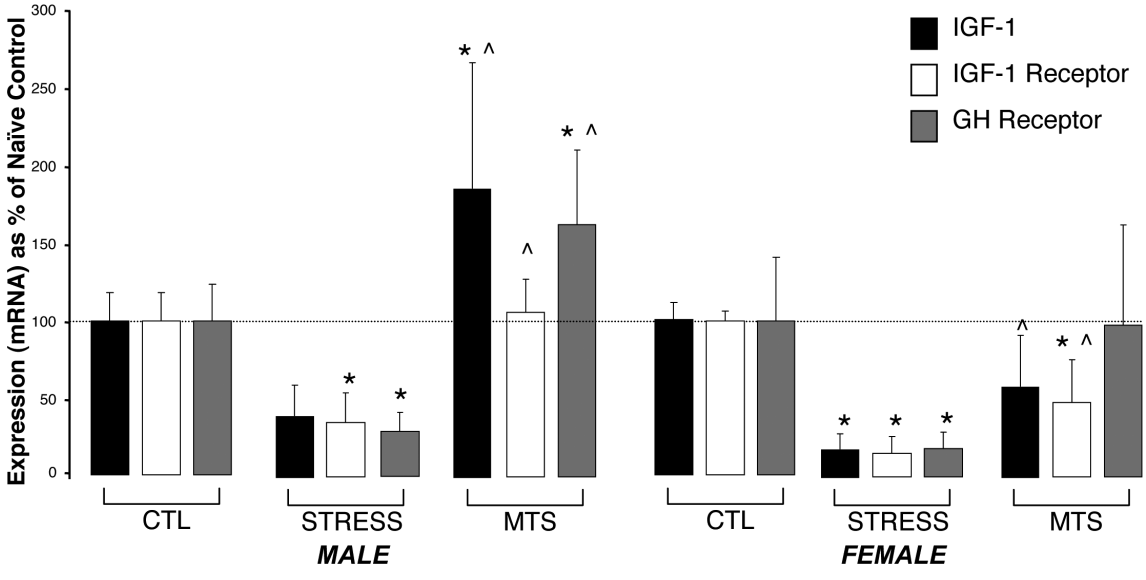

Figure 1. Bone specific levels of mRNA for Insulin-like Growth Factor 1 (IGF-1), IGF-1 Receptor, and Growth Hormone (GH) Receptor from real-time RT PCR plotted as percent of naïve control (CTL). Plotted mean ±SD for neonatal stress control (STRESS) and neonatal stress with mechanical/tactile stimulaiton (MTS). *denotes significance (p≤0.05) compared to naïve CTL and ^ denotes significance (p≤0.05) compared to STRESS.

and STRESS (Fisher's LSD p=0.04). MTS females had greater hypertrophic width than naïve CTL (Fisher's LSD p=0.001) despite similar growth plate width. Females had smaller growth plate width than males ($ANOVA_{sex\ effect}$ p=0.013) but there was no difference in hypertrophic zone width between sexes ($ANOVA_{sex\ effect}$ p=0.07).

Bone densitometry by Dual X-Ray Absorptometry (DXA)

Neonatal stress in our model has a long term impact on bone with neonatal stress groups (both MTS and STRESS) having over 20% less bone mineral content (BMC) and 40% less bone area (BA) than naïve control (CTL) adolescent rats ($ANOVA_{treatment\ effect}$ p≤0.001; Table 1). However, male rats that received MTS had 11% higher bone mineral (BMC) than STRESS males (Table 1; Fisher's LSD p=0.032). Overall, males had greater BMC and BA than females ($ANOVA_{sex\ effect}$ p≤0.001, Table 1). Apparent bone mineral density (aBMD) is calculated as the amount of BMC (grams) per BA (cm^2). Because of the smaller BA, both neonatal stress groups had greater than 10% higher calculated aBMD compared to naïve CTL. MTS treatment had approximately 7% higher aBMD than STRESS as a result of higher BMC ($ANOVA_{treatment\ effect}$ p≤0.001; Table 1). There was a significant interaction effect (p=0.005) explained by a dif-

	Osteocalcin (ng/mL)	Propeptide of type I collagen, PINP (ng/mL)	Tartrate resistant acid phosphatase, TRAP (U/L)	Insulin-like Growth Factor-1 (ng/mL)	IGF Binding Protein 3 (ng/mL)
Male					
CTL	151±13	21.5±5	6.1±2.1[c]	549±77	0.94±0.41
STRESS	160±7	20.8±4	6.5±1.3[c]	491±110	1.10±0.44
MTS	191±23[a,b]	18.9±4	4.4±0.5	425±110	1.07±0.23
Female					
CTL	155±10	7.3±2	5.6±0.8	332±50	0.70±0.33
STRESS	171±8	9.0±3	5.0±0.8	455±55	0.85±0.23
MTS	202±16[a,b]	10.0±3	6.0±0.8	403±58	0.41±0.03

Data are expressed as mean ±SD: Naïve control (CTL); Neonatal stress control (STRESS); Neonatal stress with mechanical/tactile stimulation (MTS). Where significant (p≤0.05) the letters signify [a]: >STRESS; [b]: >CTL; [c]: >MTS.

Table 2. Serum bone turnover markers, IGF-1, and IGFBP3 levels by treatment and sex for Sprague-Dawley rats at day 60 of life.

ference in BMC that was only significant in males by Fisher's LSD pairwise comparison where MTS males had higher BMC than STRESS (p=0.015).

Serum bone turnover markers

Neonatal MTS resulted in approximately 18% higher circulating osteocalcin, a marker of bone formation, in both males and females compared to both CTL and STRESS groups ($ANOVA_{treatment\ effect}$ p=0.001, Table 2). Another marker of bone formation, propeptide of type I collagen (PINP), was not different among treatment groups but was higher in male rats than female rats ($ANOVA_{sex\ effect}$ p≤0.001, Table 2). MTS treatment in males resulted in approximately 30% lower TRAP, a bone resorption marker, than both CTL (p=0.03) and STRESS (p=0.01) males by Fisher's LSD pairwise comparison ($ANOVA_{interaction\ effect}$ p=0.014; Table 2).

Serum IGF-1 and IGFBP-3

The liver is the primary source of circulating IGF-1. The majority of circulating IGF-1 is carried within a complex that includes IGF binding protein 3 (IGFBP-3). IGFBP-3 has a stabilizing effect on IGF-1 in circulation. Serum levels of IGF-1 and IGFBP-3 were not different across treatment groups (Table 2). IGFBP3 was higher in males than females ($ANOVA_{sex\ effect}$ p=0.004).

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

Bone specific IGF-1 mRNA levels resulted in significant treatment (p≤0.001) and interaction effects (p=0.004). Bone-specific IGF-1 mRNA levels were about 4.5 times higher in MTS males (Fisher's LSD p≤0.001) and about 3 times higher in MTS females (Fisher's LSD p=0.05) than sex matched STRESS (Figure 2). Compared to CTL, males and females of the stress groups (STRESS and MTS) responded differently. Bone IGF-1 was higher in male MTS than male naïve CTL (Fisher's LSD p=0.003) whereas STRESS females had lower

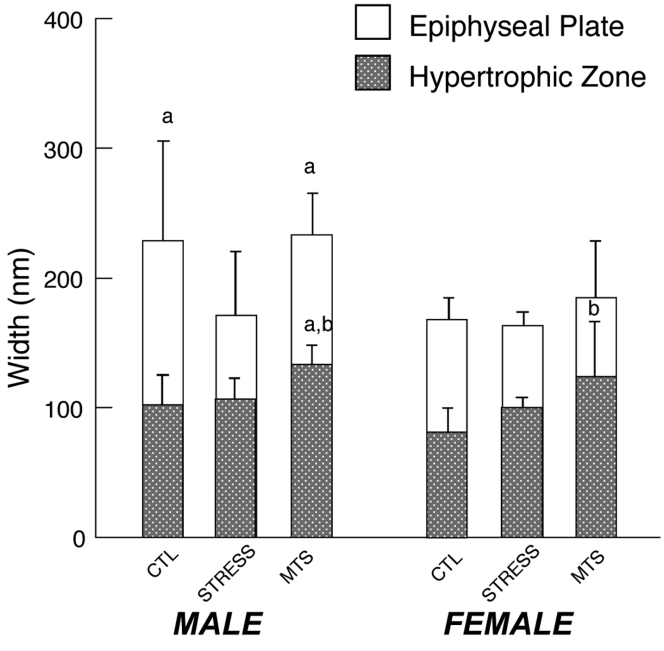

Figure 2. Epiphyseal plate morphometry and hypertrophic zone plotted mean width ±SD for naïve control (CTL), neonatal stress control (STRESS) and neonatal stress with mechanical/tactile stimulaiton (MTS). Where significant (p≤0.05) the letters signify a: >STRESS; b: >CTL; c: >MTS.

IGF-1 than female naïve CTL (Fisher's LSD p=0.004). MTS females and STRESS males were not different from naïve CTL (Figure 2). IGF-1R mRNA also revealed a significant treatment effect (p≤0.001) and interaction effect (p=0.007). MTS males were similar to naïve CTL while STRESS males were more than 3 times lower (Fisher's LSD p≤0.001). Females in the neonatal stress groups (STRESS and MTS) had significantly lower levels of IGF-1R mRNA than naïve CTL females

(Fisher's LSD p=0.001). IGF-1R in female STRESS is 6 times lower than female naïve CTL (Fisher's LSD p≤0.001) and 3 times lower than female MTS (Fisher's LSD p=0.03). GHR mRNA was 5 times higher in naïve CTL and MTS than STRESS ($ANOVA_{treatment\ effect}$ p<0.001; Figure 2). Hepatic expression of IGF-1 and IGF-1R mRNA levels were not different among treatment groups (data not shown).

Discussion

Stressful stimuli during critical periods of early development exert a prolonged negative impact that results in poor quality bone. Evidence suggests that interventions with mechanical/tactile stimulation (MTS) may benefit bone[11,12,30]. We find evidence that neonatal MTS may exert a long-term impact on bone in a model for neonatal stress and that the IGF-1 axis may be a participating mechanism. Results from this research also support the hypothesis that MTS intervention attenuated the negative impact of neonatal stress on bone, particularly in male rats. Male rats that received neonatal MTS intervention had improved BMC and aBMD as well as serum markers that suggest reduced bone resorption of osteoclasts and increased formation by osteoblasts compared to STRESS. The accompaniment of lower osteoclast and higher osteoblast activity in MTS males is indicative of greater overall bone formation. Increased bone formation is supported by DXA data demonstrating that MTS adolescent male rats have greater BMC and aBMD. It was also predicted that the IGF-1 axis is a participating mechanism in the disruption of bone development in this neonatal stress model and central to improvements associated with MTS. MTS resulted in tibia mRNA expression levels of IGF-1, IGF-1R, and GHR that more closely resembled that of naïve CTL while STRESS mRNA were reduced several fold. In the case of IGF-1, mRNA expression levels exceeded that of naïve control in males. In summary, MTS appears to attenuate the negative impact of early life stress on bone development and IGF-1 may be a participating mechanism in this attenuation.

The IGF axis is a critical regulator of bone that can modulate the differentiation and activity of osteoblast and osteoclast cell lineages[31,38]. IGF is the most abundant growth factor produced by osteoblasts and stimulates mature osteoblast function and increases bone matrix formation while decreasing degradation[31]. Mice lacking IGF-1 are deficient in bone formation and have a 60% deficiency in bone mineral density[39-41]. In human cases of IGF-1 gene disruption there is also a several fold reduction in peak BMD[31]. Growth impairment is derived primarily from reduced local or bone specific IGF-1 rather than hepatic derived IGF-1 in circulation[42]. Transgenic studies that result in decreased circulating IGF-1 indicate that systemic IGF-1 is dispensable for postnatal linear growth. Our data find hepatic and circulating IGF-1 unchanged yet bone specific IGF-1 is altered and may be more important for bone mineralization and growth. The higher levels of mRNA do not necessarily correspond with protein expression and translational upregulation will need to be confirmed.

It is argued that neonatal MTS decreases the negative impact of environmental stressors on growth and bone development by reducing the autonomic stress response. *In vivo* exposure to glucocorticoids results in bone loss and a down-regulation of IGF-1[34]. Stress during critical periods of development diminishes IGF-1 and slows growth and development in infants and adolescents[34,43-46]. Preterm infants that receive massage therapy that incorporates tactile stimulation and kinesthetic movement have increased weight gain as well as circulating IGF1 levels and reduced levels of stress hormones[8,35]. Animal models have found that stress response is ameliorated by tactile interventions. Supplemental stroking or increased maternal licking and grooming improved the developmental programming in rodents and resulted in benefits to adult behavior, phenotype, and reduced anxiety response[19,21,28,29,36,47,48]. Furthermore, tactile stimulation was associated with decreases in circulating stress markers and increases in weight gain in rat pups[49-51]. In our animal model, MTS had greater weight gain than both CTL and STRESS groups. Stress tests were not performed in this study to avoid the confounding effects they would exert on our results. However, an improved response to environmental stressors is a potential means by which MTS may have a long-term impact on bone mineralization that should be explored.

The early life insult of neonatal stress and changes associated with MTS may persist into adolescents as a result of epigenetic programming. IGF-1 is an epigenetically regulated gene. The perinatal insult of intrauterine growth restriction (IUGR) results in alteration to the epigenetic characteristics of the IGF-1 gene[52]. These changes are used to explain why IUGR results in reduced IGF-1 and poor postnatal growth. Furthermore, variations in maternal care have been shown to alter epigenetic programming of stress response. Amounts of maternal licking and grooming in early life program the HPA response to stress in offspring. Offspring exhibit greater hippocampal glucocorticoid receptors and feedback sensitivity to stress[49-51,53]. Evidence suggests this is due to changes in DNA methylation patterns of the glucocorticoid receptor (GR) promoter, altering GR expression in the hippocampus and stress response[54-57].

Improvements associated with MTS may also be the result of mechanical stimulation. Mechanical stimulus is positively linked to bone formation by osteoblasts in multiple *in vivo* and *in vitro* models[58]. Dynamic mechanical stimulation in rat models attenuates bone loss. Evidence suggests that low-level bone strain and generation of fluid pressure from muscle action is osteogenic[58,59]. Furthermore, IGF-1 mRNA expression increases in rat osteocytes in response to mechanical stimulation[60]. Benefits of mechanical stimulation and improved stress response are not exclusive of each other and may work in concert to improve the IGF-1 axis.

Consistent with previous clinical studies and animal models of MTS, there appears to be a reduction in bone resorption and increased bone formation as a result of MTS. In a clinical study of MTS administered to preterm infants, MTS resulted in reduced levels of pyridinium crosslinks, a biochemical marker

of osteoclast activity, excreted in the urine compared to control infants[12]. An animal model of tactile stimulation resulted in decreased eroded surface, an indication of less bone resorption, in femur from D60 rodents compared to controls[30]. This in conjunction with the detection of increased mineralized surface indicates that reduced resorption was coupled with increased formation. Females who received tactile stimulation also had a greater mineral apposition rate reflecting greater osteoblast activity[30]. Our study found reduced TRAP, a circulating marker of bone resorption in MTS males and an increase in osteocalcin, a marker for bone formation, in both sexes. The suppression of TRAP reflects a possible reduction in osteoclast number but not necessarily activity. Chen et. al. (2009) concluded that tactile stimulation may suppress bone resorption and increase bone formation to result in a positive bone gain and connectivity of trabeculae[30]. Our study supports this theory in a model for neonatal stress. Furthermore, data indicate that MTS may improve the long-term bone mineralization trajectory by changing the dynamic of bone growth, modeling, and remodeling through the IGF-1 axis into adolescence when peak bone mass is determined. Future studies will need to assess bone strength with mechanical testing, histomorphometry, and trabecular or cortical bone microarchitecture as these are limitations to the present study.

While there was a negative impact of neonatal stress in both male and female neonatal stressed rats, there were sex differences in their response to MTS. Previous studies in a non-stress model found significantly greater femur bone strength and thicker trabeculae in D60 females who received neonatal tactile stimulation[30]. Histological measures also indicated that D60 MTS females had less bone resorption[30]. In the case of the stress model reported here a more significant impact was detected in male BMC and makers of bone resorption. Bone resorption marker tartrate resistance acid phosphatase (TRAP) was not reduced in MTS females, and this may explain why a difference in BMC was not detected in females. Female rats were not without benefit from MTS, however. Osteoblast produced osteocalcin, the hypertrophic region, and tibial length all indicate that MTS female bone development was improved compared to STRESS. IGF-1 and GHR in both the male and female rat MTS groups were elevated compared to STRESS but females did have lower IGF-1 Receptor mRNA. Sex differences may be the result of differences in hormone concentrations, timing, and duration of pubertal bone development. For example, the onset of puberty begins around D34 for female and around D65 in male rodents[61]. This difference would alter the maturation progress between the sexes and is why data is reported with sexes separated. Furthermore, growth hormone is secreted in a sexually dimorphic pattern where males release GH in discrete pulses whereas the female pattern is less pulsatile[31]. GH secretion influences the IGF-1 axis and may contribute to sex differences in mRNA expression of GHR, IGF-1 and the IGF-1 receptor.

Both GH and IGF-1 are involved in the promotion of longitudinal bone growth[31]. Long bone growth rate is determined by the rate of epiphysial growth plate chondrocyte generation and the final size of hypertrophic chondrocytes that form the scaffolding for bone elongation[31]. Growth hormone (GH) stimulates the expansion of the germinal zone and enhances chondrocyte proliferation. While growth plate width was larger in naïve CTL and both growth plate and hypertrophic zone were larger in MTS male rats compared to STRESS males, there were no differences detected in tibia length. Methods of measuring tibia length have large variation and the small sample size (n=6/sex/treatment) may have reduced the likelihood of finding a difference. While differences in tibia length were not detected, it does appear that BMC of bone may be improved. Interestingly, females show little differences in growth plate morphometry but naïve CTL tibia length was greater than STRESS. IGF-1 and GHR mRNA was elevated in both naïve CTL and MTS compared to STRESS and may explain this difference.

To our knowledge, this is the first study to investigate bone outcomes and IGF-1 response in the context of neonatal stress with a mechanical/tactile stimulation (MTS) intervention. The results identify a negative impact of neonatal stress on bone mineralization and improvements as a result of MTS intervention. The current study encourages future investigation into the role that neonatal stress and MTS may have on the epigenetic programming of IGF-1 and stress response genes. Future studies will need to assess differences in stress response and distinguish between effects associated with mechanical stimulation verses stress reduction.

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