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Original Article

Hylonome

Massage therapy during early postnatal life promotes greater lean mass and bone growth, mineralization, and strength in juvenile and young adult rats

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Abstract

The objects of this study were to investigate the effects of massage therapy during early life on postnatal growth, body composition, and skeletal development in juvenile and young adult rats. Massage therapy was performed for 10 minutes daily from D6 to D10 of postnatal life in rat pups (MT, *n*=24). Body composition, bone area, mineral content, and bone mineral density were measured by dual energy X-ray absorptiometry (DXA); bone strength and intrinsic stiffness on femur shaft were tested by three-point bending; cortical and cancellous bone histomorphometric measurements were performed at D21 and D60. Results were compared to age- and gender-matched controls (C, *n*=24). D21 body weight, body length, lean mass, and bone area were significantly greater in the MT cohort. Greater bone mineral content was found in male MT rats; bone strength and intrinsic stiffness were greater in D60 MT groups. At D60 MT treatment promoted bone mineralization by increasing trabecular mineral apposition rate in male and endosteal mineral surface in females, and also improved micro-architecture by greater trabeculae width in males and decreasing trabecular separation in females. In summary, massage therapy during early life elicited immediate and prolonged anabolic effects on postnatal growth, lean mass and skeletal developmental in a gender-specific manner in juvenile and young adult rats.

Keywords: Massage Therapy (MT), Body Composition, Bone Growth, Mineralization, Bone Histomorphometry, Bone Strength

Introduction

Gentle tactile stimulation or 'touch' and infant massage therapy have been promoted as an effective complementary intervention for stress reduction and enhancement of postnatal growth and development in term and prematurely born infants (<37 weeks gestation)[1,2]. Massage therapy focuses on the skeletal and soft tissues of the body and uses hand-applied force and movement to general or specific areas of the body with the goal of assisting the body in self-regulation and healing.

Recent studies of noninvasive forms of stimulation such as massage have generally reported improvements in growth and/or behavioral development[3,4]. Supplemental tactile/kinesthetic stimulation studies performed in preterm infants[5,6] have shown enhanced growth and bone mineralization, less stress behavior, and better performance on developmental assessments at 8 months of age[7-12]. Kuhn et al.[13] found that massage that incorporates both tactile stimulation and kinesthetic movements enhanced postnatal weight gain and neural behavior in preterm infants also facilitated a normal developmental rise in catecholamine excretion. Specifically, integrated sampling under non-stressful conditions demonstrates a highly stable individual pattern of catecholamine and cortisol activity during early human development. Similar findings have been reported in term newborns[1]. Thus massage therapy during early postnatal life appears to temper stress and promote growth and development in newborn infants.

Rodent models provide strong evidence of the potential for massage therapy to modulate the neuroendocrine response to stress. In animals, postnatal stress such as maternal separation, repetitive pain, or undernutrition in early life may permanently

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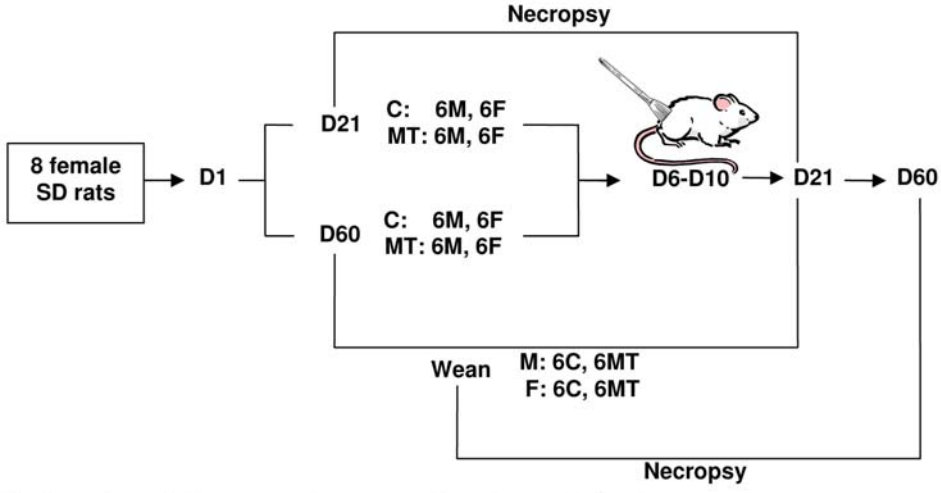

Figure 1. The flow chart for experiment groups and time point.

change body structure, physiology, and metabolism increasing the incidence of adult chronic diseases. Maternal separation during early postnatal life disrupts maturation of the adrenal stress response[14], alters cortisol expression, and negatively impacts adult learning, long-term potentiation, and hippocampal synaptic organization[15]. Similar to humans, undernutrition during critical time periods around birth in rat pups has long-lasting effects on body composition and skeletal growth[16]. "Massage-like" stimulation during early postnatal life improves ANS balance and tone[17-20], pain control[21], and weight gain[22] and decreases behavioral and endocrine response to stress in adult animals[22]. Most recently, Chatterjee et al.[17] elegantly demonstrated that daily "massage-like" stimulation during early postnatal life reversed altered neural protein expression elicited by extreme maternal isolation.

The impact of massage therapy during early postnatal life on bone tissue growth and development has not been addressed in a neonatal animal model. Therefore, we investigated whether massage therapy during early postnatal life affects postnatal growth and patterns of skeletal growth, bone mineralization, and bone strength.

Material and methods

Animal & material

Eight female, 3-month-old Sprague-Dawley rats (Charles River, Wilmington, MA, USA) with timed pregnancies were allowed to deliver at term (gestation=21.5 days). Each rat dam was housed individually (58cm × 36cm × 20cm cage) at room temperature (72°F) with a 12-hour light/12-hour dark cycle. All dams were allowed free access to water and a pelleted commercial natural diet (Teklad Rodent Laboratory Chow #8640, Harlan Teklad, Madison, WI) containing 0.95% calcium, 0.67% phosphorus, and 4.5 IU/g vitamin D_3. Cages were inspected for birth each morning and the date of birth was recorded as the previous day unless it was apparent that the litter had recently been born. One day after birth (D1), rat pups were initially weighed and culled to create weight-matched, sex-balanced litters. Mortality, weights and body size were monitored at intervals throughout the experiment. Rat pups were weaned and separated by sex at age D21. At necropsy (D21 or D60), the animals were anesthetized with Avertin (1ml/100g) and killed by cardiac puncture (Figure 1). All animal treatments were conducted according to a University of Utah Institutional Animal Care and Use Committee-approved protocol and the animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

Treatment groups

Litters were randomly assigned to one of two treatments (1) C for control (dam reared, *n*=24 pups, 12 males, 12 females) and (2) MT (n=24 pups, 12 males, 12 females). The MT intervention, modified from Meaney et al.[23] and other animal massage studies[17-20]. MT consisted of 10 minutes of stroking from head to tail in ventral and dorsal positions with a soft camel hair brush, moistened with warm water to mimic maternal licking and grooming. The MT intervention began on D6 and continued daily for 5 days until D10. The MT pups were removed individually to receive treatment and immediately returned to their home cage at the end of the 10 minute treatment session. All other handling of either MT or Control pups was limited to obtaining physical measurements or cage cleaning.

Physical measurements

Physical measurements were collected in order to monitor somatic growth. These measures included body weight (g) after each treatment session, weekly body weight (g) and size

(body length of the truck, cm), and daily weight of food (g) and water (ml) intake consumed by per animal from D21 (weaning) to D60.

Dual energy X-ray absorpitometry (DXA)

Body composition and one variable were measured one day prior to necropsy (D20 or D59). Measurements were performed by a peripheral dual energy X-ray absorptiometry (pDXA; Norland, Medical Systems, Fort Atkinson, WI) with the animal sedated by isophorene anaesthesia. The DXA, calibrated for small-animal research, assessed body lean mass (g), fat mass (g), bone area (BA, cm^2), bone mineral content (BMC, g), and bone mineral density (areal BMD, g/cm^2)[24] in the area of the truck from sternum to coccyx. The daily coefficient of variation for the manufacturer-supplied phantom was 0.6%. The precision for the DXA measurements was estimated by duplicate measurements at the same time point, the CV for repeat scans and standards was <1.0%.

Bone strength detected by three-point bending technique

Three-point bending is useful for measuring the mechanical properties of the bone from rodents and other small animals[25]. Mechanical testing to assess bone strength of the femoral midshafts was carried out on all groups. At the necropsy, the right femur was wrapped in saline-soaked gauze and immediately frozen and stored at -70°C. The femurs were completely thawed at room temperature prior to three-point bending, remaining in the saline gauze. Femur length and diameter of the femoral shaft were recorded using vernier calipers (Mitutoyo, Japan). Femurs were placed under a gradually increasing load until fracture (MTS, Eden Prairie, MN). The MTS is equipped with a 5-kN load cell and the femur bone was loaded to failure by three-point bending at a loading rate of 10 mm/min[25-27]. The load was measured with a load cell connected to a computer via an amplifier and the load-deformation curves were recorded online in TestStar IV (MTS Systems Corp., Eden Prairie, MN). Peak load, break load, extrinsic stiffness, and work to fracture were measured from the load-deformation curve[26].

Bone histomorphometry

All pups were injected subcutaneously with 10 mg/kg Calcein (Sigma Chemical, St. Louis, MO) on -4 and -1 days prior to necropsy. At necropsy the left femur and tibia were harvested and prepared for bone histomorphometric analysis. The tibiae were sliced in half through the mid-diaphyseal shaft and fixed for 24 hours in 0.1 mol/L phosphate buffered formalin. The bone tissues were then dehydrated in ethanol and embedded in methyl methacrylate (Fisher, Los Angeles, CA). Frontal sections of the proximal tibia and cross sections at the tibiofibular junction were cut on a low speed saw (Isomet, Buehler, Lake Bluff, IL), mounted on plastic slides and ground to ~20 μm in thickness using a grinding machine (Exact, Norderstedt, Germany).

Cortical bone histomorphometric indices were measured at the tibiofibular junction as previously described[28]. The primary indices included the total periosteal and endocortical perimeter (mm), bone area (mm^2), marrow area (mm^2), periosteal and endocortical single- and double-labeled surface (sLS & dLS, mm), and interlabel width (μm). The percentage of cortical area (%Ct.Ar), percentage of mineral surface (%MS), mineral apposition rates (MAR, μm/d) and surface-referent bone formation rates (BFR/BS, $\mu m^3/\mu m^2/d$) were calculated. The histomorphometric nomenclature conforms to recommendations by Parfitt et al.[29,30].

Microarchitecture and dynamic histomorphometric data were measured in the cancellous bone of the proximal tibial metaphysis, as previously described[31]. A 2-mm^2 area of cancellous bone in the proximal tibial metaphyseal secondary spongiosa was quantified using a digitizing tablet and a fluorescence microscope (Nikon, Tokyo, Japan) with histomorphometry software (KSS Scientific Consultants, Magna, UT). The proximal boundary of the measured area was defined as the junction of the primary and secondary spongiosa. The primary indices included the total tissue area (mm^2), trabecular bone perimeter (mm), trabecular bone area (mm^2), single- and double-labeled surface (mm), and interlabel width (μm). The percentage of bone area (%B.Ar), trabecular thickness (Tb. Th, μm), number (Tb. N, #/mm) and separation (Tb.Sp, μm), and percent resorption (eroded) perimeter (%E.Pm), the percentage of mineralized surface (%MS), mineral apposition rate (MAR, μm/d), and surface-referent bone formation rate (BFR/BS, $\mu m^3/\mu m^2/d$) were calculated as described by Parfitt et al.[29,30].

Statistical analysis

All data are presented as mean±SD. The means and standard deviations were calculated for all parameters from all groups. The interaction effect of treatment (MT or C) and sex (male or female) was first assessed by two-way ANOVA and then followed by Fisher's protected least significant difference post-hoc test to identify the main effects for treatment or sex (SPSS 14.0, SPSS Inc. Chicago, Illinois). As the MT intervention did significantly increased soft tissue lean mass both at D21 and D60, this variable was treated as a co-variant in the one-way ANCOVA to evaluate the significant treatment differences from controls. Rationale for using covariates is based on literature identifying lean mass/muscle as influential factors on the growing skeleton[32-37]. Probabilities (p) less than 0.05 were considered significant.

Results

The result of two-way ANOVA indicated significant interactions between MT intervention and sex for DXA- derived bone mineral content and bone area only (Figure 2). For simplicity of presentation, results reflect significant treatment effects (MT compared to C) generated by either ANOVA or ANCOVA analysis.

Growth and body composition

The mean body weight at the beginning of the experimental period (D6) was similar for all treatment groups (12.92±0.57 g vs. 12.98±0.86 g) and between males and females (12.88±0.56 g

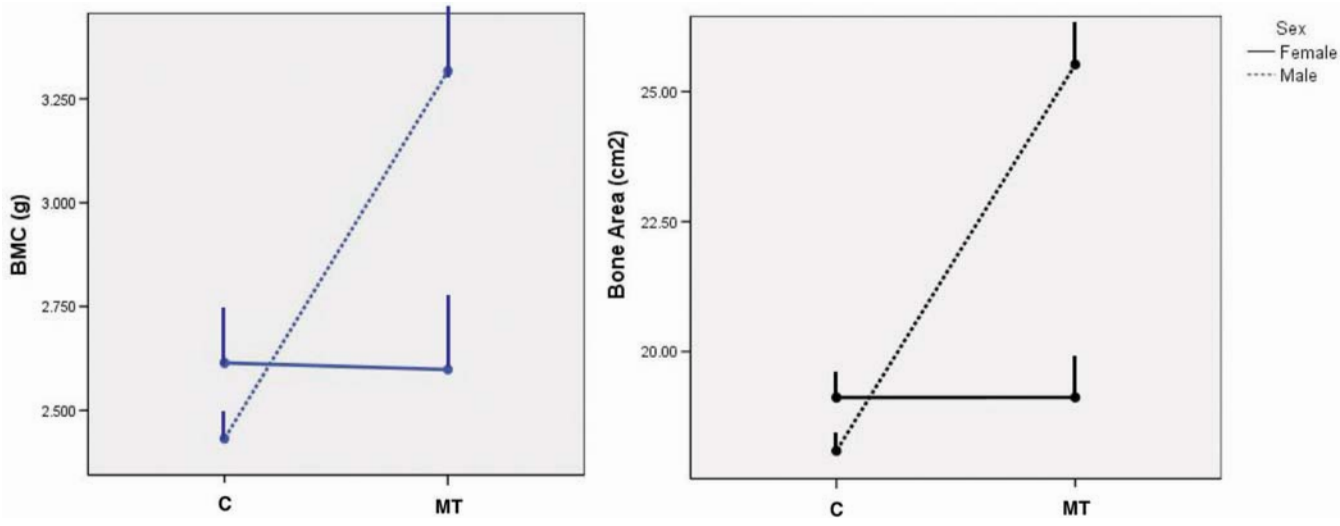

Figure 2. The plots for the significant interaction effects of bone mineral content and bone area at D60.

	Body Weight (g)	Body Length (cm)	BA (cm^2)	BMC (g)	BMD (g/cm^2)	Lean Mass (g)	Fat Mass (g)
Male							
D21-C	61.31±2.52	8.02±0.08	7.42±0.73	0.34±0.04	0.046±0.003	35.37±1.42	3.04±1.33
D21-MT	65.05±2.68 *	8.27±0.12 *	11.72±0.81*	0.56±0.05*	0.048±0.004	49.27±2.89*	3.12±0.90
D60-C	342.80±21.95	18.16±0.32	18.09±0.41	2.43±0.10	0.134±0.004	214.78±6.47	5.56±1.97
D60-MT	360.00±27.93	18.13+0.21	25.11±2.43*	3.29±0.30*	0.134±0.008	273.19±21.44*	5.72±2.10
Female							
D21-C	57.82±1.11	7.96±0.05	7.86±1.74	0.41+0.10	0.047±0.005	35.44±2.45	3.05±1.00
D21-MT	60.68±3.42	8.15±0.12*	11.19±1.57*	0.53±0.06*	0.048±0.002	43.94±2.90*	4.28±2.32
D60-C	231.40±7.09	15.96±0.36	19.12±0.99	2.61±0.14	0.137±0.006	161.40±6.41	3.29±0.76
D60-MT	238.00±8.09	16.00+0.71	19.12±1.63	2.60±0.25	0.132±0.007	175.46±10.97*	2.23±1.62

Data are expressed as mean±SD. C: Control group; MT: message therapy group; BA: bone area; BMC: bone mineral content; BMD: bone mineral density. *p<0.05 vs.C. by ANOVA.

Table 1. Physical development at D21 and D60 by treatment and sex.

vs. 13.02±0.68 g). At D21, MT pups were significantly longer than C pups (p<0.01) and MT males heavier than C males (+14.5%, p=0.04). MT pups were also found to have greater lean mass compared to C both at D21 (male: +39%, female: +24%, p<0.01) and D60 (male: +27%, female: +9%, p=0.01). No differences were identified for body weight, length or fat mass between groups at D60 (Table 1). The food and water consumption relative to body weight from D21 to D60 did not differ between the two treatment groups or by sex (data are not shown).

Bone mineral density, area, and mineral content

At D21 BA, BMC and lean mass was significantly higher in MT males than C males (BA: +58%, BMC: +66% and lean mass: +39%, p<0.01); only bone area was significantly greater in MT females compared to C females (+42%, p<0.01) (Table 1). D60 MT males continued to have larger bone area, mineral content, and lean mass versus C males (+39%, +35%, and +27%, respectively, p<0.01); lean mass was also significantly greater in D60 MT females than C females (+9%, p=0.02). No differences were detected for BMD or percent fat mass between groups or by gender at D21 or D60.

Given their greater body weight (+43.3%, p<0.01) and lean mass (+45.2%, p<0.01) it is not surprising that D60 males had greater bone mineral content (+10%, p<0.05), bone area (+13%, p<0.05) and the femur shaft diameter (+12.3%, p<0.01) and length (+10%, p<0.01) compared to females. A significant treatment and sex interaction effect was detected for bone mineral content (p=0.01) and bone area (p<0.01) at D60 (Figure 2).

	Diameter (mm)	Length (mm)	Peak Load (N)	Intrinsic Stiffness (N/mm)
Male				
D21-C	2.46±0.10	16.85±0.99	21.43±2.51	63.26±15.75
D21-MT	2.70±0.14#	17.60±0.57	20.55±3.15	60.42±9.90
D60-C	4.45±0.15	32.37±0.41	81.88±4.72	120.74±13.77
D60-MT	4.66±0.30	32.88±0.53	85.98±7.20	148.77±18.20*
Female				
D21-C	2.42±0.09	17.26±0.18	23.04±2.18	76.66±6.77
D21-MT	2.77±0.13#	17.50±0.34	21.38±2.68	70.05±10.62
D60-C	3.89±0.12	30.13±0.59	75.11±3.48	152.86±18.77
D60-DMT	4.21±0.27*	30.87±0.78	87.15±5.26*	171.13±27.51

Data are expressed as mean±SD. C: Control group; MT: message therapy group; Diameter: femur shaft diameter; Length: femur shaft length. *p<0.05 vs.C. by ANOVA. #p<0.05 vs.C by ANCOVA, adjusted for soft tissue lean mass.

Table 2. Bone mechanics variables for the femur midshaft at D21 and D60.

	Periosteal Surface				Endosteal Surface		
	%Ct.Ar (%)	**%MS (%)**	**MAR (µm/d)**	**BFR/BS (µm³/µm²/d)**	**%MS (%)**	**MAR (µm/d)**	**BFR/BS (µm³/µm²/d)**
Male							
D21-C	54.91±2.06	98.55±0.90	19.25±2.76	16.48±5.29	35.26±8.47	5.06±0.7	1.78±0.44
D21-MT	55.39±1.91	96.31±2.60	21.02±4.24	19.69±3.46	32.96±7.65	5.76±0.73	1.94±0.54
D60-C	78.85±1.94	93.06±1.43	9.24±1.61	9.11±0.93	74.70±3.36	4.68±0.59	3.47±0.40
D60-MT	78.83±3.16	93.27±3.02	11.26±1.99	10.31±2.04	81.82±7.11	4.39±0.57	3.67±0.71
Female							
D21-C	51.77±1.82	95.01±2.15	17.85±2.56	16.98±2.63	36.61±6.8	5.05±0.81	1.82±0.41
D21-MT	51.07±0.88	96.19±5.05	18.85±5.40	17.92±5.24	38.57±10.46	6.17±0.27	2.36±0.89
D60-C	76.76±1.80	95.98±3.20	8.55±1.34	8.44±1.28	81.30±3.84	4.61±0.86	3.82±0.51
D60-MT	75.21±2.18	96.62±2.95	9.11±2.33	8.75±2.15	92.84±3.75*	4.28±0.91	4.03±1.03

Data are expressed as mean±SD. C: Control group; MT: message therapy; %Ct.Ar: percent cortical area= (total area - marrow area)/total area *100; %MS: percent mineralized surface= (dLS+sLS/2)/periosteal or endosteal perimeter * 100; MAR: mineral apposition rate= interlabel width/days; BFR/BS: bone surface referent bone formation rate MS * MAR/periosteal or endosteal perimeter. *p<0.05 vs.C. by ANOVA. #p<0.05 vs.C. by ANCOVA, adjusted for soft tissue lean mass.

Table 3. Cortical bone histomorphometric variables of the tibia shaft at D21 and D60.

Bone strength

At D21, the diameter of the femur shaft was 10% greater in MT males and 14% greater in MT females than C (p<0.01). At D60, The intrinsic stiffness was significantly higher in MT males compared to C males (+23%, p=0.02); MT females were found to have greater femur diameter (+8%, p=0.04) and an increased peak load (+16%, p<0.01) when compared to C females (Table 2). Females had higher intrinsic stiffness (+18.7%, p=0.01) at D21, whereas D60 males had greater femur shaft diameter (+12.3%, p<0.01) and length (+10%, p<0.01) compared to females.

Histomorphometric profile

Tibia Shaft Cortical Bone. Neither periosteal or endosteal mineral apposition or bone formation rates differedbetween D21 cohorts. However, MT females were found to have a greater percent mineral surface on the endosteal surface when compared to C females (+13%, p=0.02) at D60 (Table 3). At D21, males had greater cortical bone area (8%, p<0.01) in the tibia shaft, and female rats had greater endosteal mineral surface (+11.6%, p<0.05) in the tibia shaft at D60.

Microarchiecture in the Tibia Primary Metaphysis. At D60, trabeculae width was significantly greater (+16.9%, p=0.04) in MT males compared to C males, and MT females had less trabecular separation (-21.6%, p=0.04) than C females (Table 4). No differences were found between treatments or by gender at D21. At D21, males had thicker trabeculae (+26%, p=0.01) in the primary tibia metaphysic than females.

Histomorphometric Profile of Trabecular Bone in the

	%B.Ar (%)	Tb.Wi (µm)	Tb.N (#/mm)	Tb.Sp (µm)
Male				
D21-CTL	12.83±4.49	47.39±6.93	2.55±0.76	362.23±109.95
D21-DMT	11.45±2.74	42.33±3.15	2.71±0.65	329.81±71.03
D60-CTL	17.16±3.72	49.21±4.03	3.58±0.63	270.19±81.21
D60-DMT	19.98±6.97	59.24±8.80*	3.45±0.96	242.65±70.94
Female				
D21-CTL	10.23±2.61	36.13±4.75	2.83±0.57	328.73±74.33
D21-DMT	10.00±1.71	35.11±2.44	2.78±0.51	336.02±82.19
D60-CTL	21.53±3.39	55.30±8.85	4.17±0.45	210.86±39.09
D60-DMT	26.96±5.70	59.23±12.87	4.58±0.82	165.29±47.33*

Data are expressed as mean±SD. C: Control group; MT: message therapy. %B.Ar: percent trabecular area=bone area/total tissue area * 100; Tb.Wi: trabecular width=(2000/1.199)* bone area/bone perimeter; Tb.N: trabeculea number=(1.199/2)* bone perimeter/tissue area; Tb.Sp: trabecular separation=(2000/1.199)* (tissue area - bone area)/bone perimeter. *p<0.05 vs.C. by ANOVA. #p<0.05 vs.C. by ANCOVA, adjusted for soft tissue lean mass.

Table 4. Cancellous bone microarchitectural variables in tibia metaphysis at D21 and D60.

	%E.Pm (%)	%MS (%)	MAR (µm/d)	BFR/BS (µm³/µm²/d)
Male				
D21-C	6.52±1.37	39.03±6.35	3.93±0.34	1.18±0.26
D21-MT	6.04±1.19	35.27±4.72	3.80±0.45	1.07±0.20
D60-C	6.94±1.77	38.63±2.92	4.23±0.17	1.35±0.12
D60-MT	6.56±2.47	42.33±5.08	5.01±0.40*	1.69±0.33
Female				
D21-C	6.26±1.51	38.12±4.70	3.67±0.56	1.07±0.26
D21-MT	7.91±0.65	40.46±3.91#	3.63±0.27	1.16±0.13#
D60-C	5.05±2.81	33.31±3.18	4.73±0.48	1.31±0.24
D60-MT	5.26±1.23	38.90±5.38	5.33±0.81	1.68±0.41

Data are expressed as mean±SD. C: Control group; MT: message therapy; %E.Pm: percent eroded perimeter=eroded perimeter/bone perimeter * 100; %MS: percent mineralized surface=(dLS+sLS/2)/ bone perimeter * 100; MAR: mineral apposition rate=interlabel width/days; BFR/BS: bone surface referent bone formation rate MS * MAR/bone perimeter. *p<0.05 vs.C. by ANOVA. #p<0.05 vs.C. by ANCOVA, adjusted for soft tissue lean mass.

Table 5. Cancellous bone histomorphometric variables in tibia metaphysis at D21 and D60.

Tibia Primary Metaphysis. At D21 percent mineral surface (+6%, p=0.03) and bone formation rate (+8%, p=0.01) was greater on the cancellous bone surface in MT females. At D60 the mineral apposition rate was greater in the MT males (+18%, p<0.01) than C males (Table 5).

Discussion

To our knowledge this is the first report of the impact of massage therapy during early postnatal life on skeletal growth and development in an animal model. Consistent with the previous clinical studies[10,11,38-43], the current study demonstrated that massage therapy during early life was associated with greater weight gain, lean mass, and bone growth, mineral acquisition, and strength. Previous clinical or animal studies demonstrate positive effects on growth and neurodevelopment from massage used as a treatment to temper moderate to severe stress associated with premature birth or maternal-separation conditions. Our results confirm the efficacy of massage therapy in the presence of minimal stress during early life in relation to growth, body composition, and bone development in juvenile and young adult rats.

Body weight, length, and lean mass at weaning (D21) were greater in both male and female rats after receiving daily MT during early life (D6-D10). We attribute these growth and body composition differences to the massage intervention as all animals were housed in the same environment throughout the

study period. We cannot, however, eliminate variations in maternal care or response to separation between MT and Control dams as a potential confounder variable. Animal and human studies of stress during the neonatal period have reported an inverse association between weight gain and levels of cortisol or epinephrine[5,7,8,37,42,44-47]. It is postulated that massage modulates the autonomic nervous system's response and recovery to environmental stressors thus minimizing the negative impact of stress on postnatal growth[5,7,8,35,42,44-47]. In premature infants, massage including touch with kinesthetic movement, has been linked to greater weight gain, decreased cortisol levels, lower blood pressure, and increased gastric motility and the release of GI hormones[4,7,8,37]. Somatic growth is controlled by pituitary gland-derived growth hormone (GH), which stimulates insulin-like growth factor 1 (IGF1) activity in the liver, bone, muscle, and other tissues. The GH/IGF1 axis activity is greatest during the early postnatal period and again during adolescence. Physical and environmental stressors have been shown to diminish GH/IGF1 activity during critical periods of growth and development in infants and young animals. Higher IGF1 levels and bone mineral deposition have been reported in premature infants who received a daily MT during early life[42]. Although positive effects of MT on lean mass acquisition and subsequent greater bone mineral acquisition were documented, other factors or the mechanism(s) responsible for this finding were not explored in the current study. Thus, exploration of the association of MT and GH/IGF1 may help define mechanisms for MT and weight gain, body composition, and skeletal growth and development.

Greater bone dimensions in MT rats were further verified by physical measures of the femur size and bending strength. A significantly larger femur mid-shaft diameter and length was found at D60 in MT males and was positively correlated with DXA-derived bone area (r=0.91, $p<0.01$). Although no differences in femur bone strength were detected at weaning we did find significantly greater femur bone strength in D60 MT females. The absence of a similar finding in D60 MT males may reflect differences in the timing and tempo of pubertal-driven skeletal growth and expansion[48], with puberty achieved at an earlier age in female rats than in males. More specifically, sexual maturation is attained at the beginning of D34 of life in female rats whereas the males do not complete pubertal-driven skeletal growth and maturation until D65.

Interestingly, D60 MT male rats were larger and had greater femur bone size whereas D60 MT females displayed higher peak load in the three-point bending test Periosteal bone formation can be a compensatory mechanism to maintain bone strength in situations where bone loss occurs from trabecular and endocortical surfaces[49]. Periosteal expansion significantly increases bone strength[50]. The relationship between periosteal dimension and bone strength is exponential; increases of periosteal radii enhance modulus (an estimator of bone strength) by the fourth power[51]. Small increases in periosteal apposition are mechanically advantageous as limited amounts of new bone can substantially increase fracture resistance and can mechanically offset loss of endocortical/trabecular bone. Estrogen has surface-specific effects on cortical bone; on the periosteal surface it inhibits the proliferation and differentiation of osteoblasts, whereas on the endocortical surface it might enhance osteoblastic activity[52,53]. Periosteal bone formation is largely due to the muscle-bone relationship, in which muscle contributes to the largest load-bearing effect[54,55].

The bone histomorphometry studies support and provide insight into the previously addressed bone growth and mineral gains observed in the MT animals. Specifically, MT during early postnatal life increased the endosteal mineral surface on cortical bone area, and the mineralized surfaces, mineral apposition and bone formation rates for cancellous bone surface in juvenile and young adult rats.

Histomorphometric evaluation of the tibia and femur bones confirmed the presence of the modeling drift that occurs during rapid skeletal expansion and mineral deposition[56]. During modeling the trabecular bone microarchitecture is improved by cancellous bone formation and consolidation. Indeed, the increased percent mineral surface, mineral apposition and bone formation rates on the cancellous bone surface at D21 MT female rats, as well as the greater percent endosteal mineralized surface and the trend of decreased trabecular spacing, confirms the development of a thicker trabeculae in D60 MT females. A trend of increased trabecular bone area or density enhances bone strength, and greater bone strength was also found in D60 MT females (Tables 2 and 4). Conversely, D60 MT males exhibited a tendency of greater trabecular width and mineral apposition rate compared to D60 C males (Table 4). It appears that MT impacted histomorphometric variables in females during juvenile period (D21) in a more sensitive manner than in males, and then developed a prolonged, positive influence of MT on bone strength at young age (D60). The absence of similar findings in MT males may be explained by puberty. During puberty the sex steroids estrogen and testosterone drive accelerated lean mass and skeletal growth by increasing GH/IGF1 axis activity. In rats, the onset of puberty occurs at a later age in males (D45) compared to females (D34)[57]. Therefore, sex differences in bone tissue development are most likely due to the timing of the onset of puberty.

During puberty, sex hormones induce an increase in the GH/IGF system to promote linear growth and bone expansion. As maturation progress, bone turnover is reduced, which increases cortical bone thickness and strength. An early study of cortical dimensions, based on two-dimensional radiogrammetry, concluded that a greater cortical bone mass in healthy boys was caused by sex differences in the rate of endosteal apposition and resorption[53]. Garn et al.[52,53] found endosteal apposition began earlier and was in greater magnitude in girls than in boys as a result of the estrogen surge at puberty. The authors postulated that female endosteal bone is accrued during puberty in anticipation of future reproductive needs and to minimize bone loss secondary to diminished estrogen levels in later life.

Examination of bone formation characteristics in young adult female rats in the current study revealed increases in the mineralizing surface for animals that received MT in early life. This finding is consistent with the decreased eroded surface, a

bone resorption parameter, after adjusting for lean mass in the same animals, indicating an increase in bone formation coupled with a decrease in bone turnover and resorption. The mineral apposition rate, a parameter reflecting the activity of osteoblasts, was also increased in the MT female cohort. It appears that MT suppressed bone resorption and increased bone formation resulting in a positive bone gain and a possible better connectivity of trabeculae. Taken together, the positive balance between bone formation and resorption and the improvement of microarchitecture contributed to the efficacy of MT on bone mass and structure. The positive findings observed in young adult female MT rats require further study to confirm potential gender-specific benefits of MT during early postnatal life on lifelong bone structure, strength, and health.

In summary, treatment of newborn rat pups with MT elicited an anabolic effect on postnatal growth and subsequent bone growth and development. MT improved lean mass deposition, stimulated bone mineral apposition on both the cortical and cancellous bone surfaces, tended to improve the microarchitecture and to decrease bone resorption on the trabecular bone surface of proximal tibia metaphysis. The current study opens a door to further study the presence of positive complementary interactions between MT in early postnatal life and the GH/IGF-1 axis, sex steroids, and the muscle-bone relationship. The lack of biochemical markers as well as absence of body composition and bone studies in older animals (>60D) suggests a need for future studies to verify a prolonged, positive impact of early life massage therapy on skeletal health.

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