MECHANICAL-TACTILE STIMULATION IN A NEONATAL STRESS MODEL ALTERS DEPOT-SPECIFIC EXPRESSION OF PROINFLAMMATORY CYTOKINES, TNF-α AND IL-6

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Premature infants are exposed to stressful events in the newborn intensive care unit (NICU). Neonatal stress is associated with increased adipose distribution to visceral depots (VAT). Adipose tissue, particularly VAT, produces two major cytokines associated with reducing insulin sensitivity, TNF-α and IL-6. Infant massage has been shown to decrease stress biomarkers. Using mechanical-tactile stimulation (MTS) as a surrogate for infant massage, we tested the hypothesis that MTS administered during neonatal stress would reduce VAT deposition as well as circulating levels and tissue-derived mRNA and protein expression of TNF-α and IL-6.

Timed pregnant dams delivered at term (E21). Litters were culled to 10 pups (5 M, 5 F) and divided into three groups: control (CTL; maternal separation), neonatal stress (NS; maternal separation + injection + hypoxia/hyperoxia) and NS + MTS (10 min of stroking and limb movement). Treatments were given from D6-10 and tissue was harvested on D120. VAT and subcutaneous (SAT) depots measures were detected using MRI and DXA. Serum levels of insulin, TNF-α and IL-6 were measured with ELISA, and cytokine mRNA levels in VAT and SAT were measured by real-time PCR. Protein expression was determined through western blotting.

MTS adult rats had a significantly lower VAT:SAT ratio and IL-6 mRNA expression in VAT depots compared to NS or CTL. Neonatal stress decreased SAT IL-6 protein expression in NS and MTS groups. TNF-α mRNA levels in VAT were significantly lower in MTS compared to NS and CTL groups. In SAT, no differences in IL-6 and TNF-α mRNA were detected and there were no detectable differences in serum levels of TNF-α and IL-6.

MTS administered during neonatal stress decreased adult VAT deposition and female insulin levels. Cytokine mRNA was lower in MTS VAT depots. Our findings suggest that early-life intervention such as MTS may have long-term positive impacts on body composition and cytokine expression.

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INTRODUCTION

Physiologic and environmental stress has negative and lasting effects on fat deposition, obesity, and development of metabolic disease [1,2]. An expanding body of research has shown that adipose tissue does more than just serve as a site for lipid storage. Adipose tissue is an active secretory (or endocrine) organ that is involved in metabolism, cell signaling, cytokine production, and hormone secretion [3,4]. Thus, adipose tissue influences physiologic and pathologic processes by responding to signals that modulate appetite, insulin sensitivity, and inflammation [4].

Adipose tissue is laid down in one of two depots: visceral (VAT) or subcutaneous (SAT). Both depots contain distinct physical properties and, more importantly, are functionally different. The SAT depot resides under the surface of the skin and primarily serves as an energy storage site. The VAT depot surrounds the organs in the abdominal cavity, including a larger retroperitoneal storage site, and is heavily involved in metabolic processes and signaling [5]. Increased proportions of VAT relative to SAT is a risk factor in the development of chronic diseases like cardiovascular disease and type 2 diabetes mellitus [3]. Prolonged, stress-induced increases in glucocorticoids lead to progressive accumulation of VAT, thereby increasing the risk of development of metabolic disease.

Adipose tissue is biologically active and evidence suggests that metabolic consequences such as insulin resistance are modulated by the release of hormones and certain proinflammatory cytokines highly correlated with obesity, namely interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα)[6,7]. Changes in adipocyte function can negatively affect insulin sensitivity via the increased release of these proinflammatory cytokines [8], independent of obesity [7]. It is known that circulating levels of these markers of inflammation are increased in insulin resistance and type 2 diabetes mellitus, and are, therefore, associated with dysregulated adipose tissue. When measured, they, along with other biomarkers like insulin and blood glucose, can be used as predictors of type 2 diabetes [9], especially in cases of excessive visceral adipose tissue [7].

Early-Life Stress

Early-life environment can play a significant role in the establishment of a phenotype that will affect adult physiology and increase risk of development of metabolic disease [10]. Premature infants (<37 weeks gestation), typically spend time in the newborn intensive care unit (NICU) in their first weeks of life where they experience repeated stressful events, including hypoxic/hyperoxic episodes, maternal separation, and painful procedures. Stress during 'critical periods' of development is associated with poor weight gain and can have long-term effects on fat distribution and development of metabolic disease [11]. At term corrected age (40 weeks) preterm infants, although shorter and lighter, are reported having greater fat mass with higher VAT depot than term-born infants [12, 13]. Thus

preterm infants provide a clinical example of how early-life stress impacts adiposity and VAT depot stores.

Adipose tissue continues to deposit preferentially to VAT stores once the episodes of stress are over [1], increasing the effects of early-life stress. It is believed that stress may cause irreversible epigenetic changes during key developmental periods of life, and the effects of stress on adiposity may not manifest themselves until later in life [14]. Prematurely born young adults have greater fat mass, abdominal VAT depot, glucose intolerance, and insulin resistance [12,15]. Although this evidence suggests early-life stress can lead to the development of life-long disease, the long-term implications of early-life stress to prematurely born infants remain unknown.

MTS

Mechanical-tactile stimulation (MTS; kinesthetic movement with massage) has been used to decrease stress in preterm infants and other newborn populations. Positive correlations have been reported between MTS, improved neuroendocrine-related stress response, and weight gain in addition to decreased hospital day in preterm infants [16]. Diego et al [17] looked at the effects of MTS on preterm infant heart rate after a painful procedure (heel stick blood draw) typical of a NICU experience. They found that MTS resulted in a faster recovery to normal heart rate after the painful stimulus compared to a control group or preterm infants who did not receive any treatment. The same group also looked at the effects of MTS in preterm infants on serum insulin and insulin-like growth factor 1 (IGF-1) levels

[17]. Both serum insulin and IGF-1 levels were significantly higher in MTS infants compared to controls. In addition, this finding was positively correlated with weight gain. In animal models of neonatal stress, MTS is reported to increase IGF-1 levels, accelerate brain and visual development, [18] and reduce anxiety-like behavior in later life [19]. Recently, Moyer-Mileur et al. [20] reported increased fat mass and abdominal SAT depot (males and females) and VAT depot (males) at weaning (D21) in a rat model of early-life stress. Importantly, they found MTS prevented hyperinsulinemia in spite of stress-induced adiposity. These authors concluded MTS during early-life stress has the potential to minimize metabolic consequences associated with stress-driven perturbations in fat mass and abdominal adipose depots. Taken together, both clinical and animal models provide evidence that MTS decreases stress and improves body composition. MTS may result in significant positive results in other aspects of health for preterm infants including growth, length of hospitalization, and improved behavior [21,22].

The purpose of this study was to determine 1) how neonatal stress affects adipose tissue deposition, fasting insulin levels, and depot-specific mRNA and protein expression of TNF-α and IL-6 in weanling and adult rats, and 2) whether MTS intervention attenuates these neonatal stress-induced changes in adult rats. We hypothesized that MTS-treated animals will have decreased VAT as well as fasting insulin and SAT and VAT expression of proinflammatory cytokines compared to neonatal stress animals.

METHODOLOGY / RESEARCH DESIGN

Animals

Timed pregnant, 3-month old Sprague-Dawley rats (n=6) were allowed to spontaneously deliver at term (E21) and litters were culled to 10 pups, 5 male and 5 female. All dams were fed a standard rat chow and housed together in the same environment. Litters will be randomly assigned to one of the three treatment groups: neonatal stress (Stress; 60 minutes of maternal separation with hypoxia/hyperoxia and a saline injection, n=20); MTS (Stress + 10 minutes of MTS at the end of the stress intervention, n=20), or maternal separation control (60 minutes of maternal separation, n=20). All interventional treatments were started on day 5 of life and were completed on day 9. Pups were cross-fostered from D5 to D20 to minimize differences in maternal care. Control pup handling was limited to daily cross-fostering (day 9-day 20) with weekly weights and cage cleaning.

Intervention

There are well-established models of neonatal stress that range from severe stress (hypoxia followed by hyperoxia, painful procedures) to mild stress (physical separation from the dam). One such model [31] was slightly modified and used which includes a 60-minute period of stressors commonly experienced by preterm infants in a NICU environment. Stress in this model refers to a 60-minute period of

maternal separation in which the pup experiences a needle puncture followed by 8 minutes of hypoxia and then 4 minutes of hyperoxia. The needle puncture, which is to simulate a painful procedure such as a blood draw or insertion of an IV catheter line, was given immediately prior to the hypoxia challenge, using a small-gauge needle. Hypoxia was achieved by placing the pups in a 22-liter, closed container kept at 37° C in a humidified gas mixture (100% N_2) for 8 minutes. At the end of the hypoxic challenge, the pups experienced hyperoxic conditions where the chamber was flushed with 100% O_2 for 4 minutes. At this time, the chamber was returned to baseline by being flushed with room air (21%O_2). The MTS pups received mechanical and tactile stimulation for 10 minutes at room air following the stress exposure but still within the 60-minute maternal separation period. To achieve mechanical stimulation, the MTS intervention consisted of 5 minutes of range-of-motion movements to the fore- and hind-limbs while tactile stimulation was provided by stroking with a soft camelhair brush to the ventral and dorsal body for 5 minutes. Equal distribution between the timing (0, 10, or 20 minutes post-neonatal stress) and research technicians among the MTS pups was assured through use of a strict rotation schedule so as to avoid bias.

Data Collection

Animals were fasted for 12 hours prior to serum collection with tissue harvested at D120 of life. Adipose tissue from both subcutaneous and visceral depots was immediately harvested after sacrifice and then flash-frozen in liquid nitrogen and stored at -80°C. Blood was collected at time of harvest in vacuum

blood collection tubes and spun in a centrifuge for 10 minutes. The separated serum was collected and stored in aliquots at -20°C until use to avoid multiple freeze-thaw cycles. Dual energy X-ray absorpitometry (DEXA) and magnetic resonance imaging (MRI) were used for quantification of body composition.

Serum levels were quantified with enzyme-linked immunosorbent assay (ELISA) for TNF-α (Abcam, Rat TNF alpha ELISA kit, ab46070), IL-6 (Invitrogen, IL-6 Rat Elisa Kit, KRC0061), and insulin (Alpco, Insulin (Rat) ELISA, 80-INSRT-E01), each according to individual protocol instructions. Detection was achieved using the Tecan Infinite F500 plate reader (Tecan Group Ltd., Switzerland). Fasting glucose was measured at time of sacrifice using the Accu-Check Aviva Glucose Meter (Roche Diagnostics) and recorded.

SAT and VAT depot IL-6 and TNF-α mRNA levels were evaluated using real-time reverse transcriptase polymerase chain reaction (RT-PCR). mRNA was extracted from crushed-frozen adipose tissue using Tissue RNeasy Lipid Tissue kit (Qiagen, DB Biosciences, CA) according to manufactures instructions. Total mRNA was quantified using the Epoch Microplate Spectrophotometer (BioTek Instruments, VT). cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster, CA) from total mRNA, and mRNA levels were determined using the comparative Ct method, with GAPDH used as an internal control. Assay-on-demand primer/probe sets were used (Applied Biosystems, Foster, CA) for both TNF-α and IL-6. For amplification, data acquisition and analysis of all real-time PCR, the 7900HT Real-time PCR system and SDS Enterprise Software (Applied Biosystems) were utilized. A 384-well Optical

Reaction Plate (Applied Biosystems) was used, and all processing was completed at the University of Utah genomics core facility. Samples were performed in quadruplicate, with cycle parameters set at: 50°C for 2 minutes followed by 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Protein expression and abundance from both retroperitoneal and subcutaneous depots and from all treatment groups was detected and compared through SDS-page gel western blot analysis. Frozen adipose tissue samples were crushed and the protein was extracted using RIPA buffer solution containing EDTA-free protease inhibitor tablets (Roch, Mannheim, Germany). The samples were mixed and allowed to rotate at 4°C for 30 minutes. The isolated protein solution was extracted, quantified in triplicate using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and stored at -80°C in 40 μl aliquots until use to avoid multiple freeze-thaw cycles of the extracts. Standard western blotting procedure was used for both TNF-α and IL-6 as follows. Gel samples containing equal amounts of total protein (40 μg) were thawed on ice and run on 10% bis-tris XT Criterion gels (Bio-Rad Laboratories, Hercules, CA) in MOPS buffer at 120 V for 100 minutes. Proteins were then transferred to PVDF membranes (Millipore, MA) with membrane blocking achieved in 3% BSA-TBST for 1 hour at room temperature following transfer. Primary antibody for both TNF-α (anti-rabbit Tumor necrosis factor alpha, ab9755, Abcam, MA) and IL-6 (anti-goat IL-6 antibody, ab9770, Abcam, MA) were mixed with the 3% BSA-TBST and incubated for 2 hours at room temperature with shaking. After thorough washing, anti-rabbit (TNF-α) and anti-goat (IL-6) secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used in 3%

BSA-TBST for 1 hour incubation at room temperature with gentle shaking. After another round of thorough washing, the signal was detected using enhanced chemiluminescence (ECL) according to the manufacture's instructions (Amersham, UK).

Statistical Analysis

SPSS software (v17.0, SPSS, Inc, Chicago, IL) was used for all analyses with $p<0.05$ considered significant. Data are presented as the mean ± SD. Post-hoc calculations were performed to determine weight gain (g/d) during and following neonatal stress. ANOVA was used to determine differences between treatments and genders, and ANCOVA analysis was done using weight as a co-factor.

RESULTS

Total body fat

Body weight as well as total and percent body fat by DXA are summarized in Table 1. Neonatal stress increased the total mass (ANOVA p=0.04) and percentage (ANOVA p=0.033) of body fat in female adult rats compared to MTS or Control groups. Male adult rat total and percentage body fat did not differ among the three treatment groups. Body weight did not differ between treatment groups but was significantly different between genders, with males having higher body weight than females (ANOVA, p=0.05).

Table 1: Body weight, total fat mass and total body fat percentage by group and gender. Data is displayed as mean ± standard deviation

Gender	Group	Body Weight (g)	Total Fat Mass (g)	Total Body Fat (%)
Male	NS	583.55 ± 24.06	55.06 ± 12.83	9.32 ± 1.97
	MTS	584.08 ± 38.5	53.01 ± 20.1	9.09 ± 3.22
	CTL	593.78 ± 13.29	57.69 ± 23.51	9.85 ± 3.8
	All Males	587.14 ± 25.28 #	55.25 ± 18.81 #	9.42 ± 3.0
Female	NS	309.68 ± 20.83	37.44 ± 12.18	11.82 ± 3.43
	MTS	299.28 ± 39.34	33.12 ± 18.19	11.36 ± 6.49
	CTL	281.88 ± 27.74	17.79 ± 4.67 * ^	6.52 ± 1.61 * ^
	All Females	296.95 ± 29.3	29.45 ± 11.68	9.9 ± 3.84

NS = Neonatal Stress, MTS = Mechanical Tactile Stimulation, CTL = Control; * $p<0.05$ vs. NS; ^ $p<0.05$ vs. DMT; # $p<0.05$ vs. All Females

Abdominal VAT depot

There were no significant differences seen in MRI total abdominal fat depot between treatment groups, although adult males had significantly more total fat than adult females. (ANOVA, p=0.01) No significant differences were seen in the abdominal VAT or SAT depot between treatment groups or by gender (Table 2). The mean VAT and SAT depots were significantly different between genders (ANOVA, p=0.001), with male animals having more abdominal VAT and SAT than females.

Table 2: MRI total abdominal, visceral, and subcutaneous adipose tissue by group and gender. Data is displayed as mean ± standard deviation.

Gender	Group	Total Abdominal Fat	VAT	SAT
Male	NS	698.13 ± 60.8	338 ± 74.75	510.93 ± 66.75
	MTS	629.1 ± 298.81	254.5 ± 143.51	539.93 ± 160.54
	CTL	818.22 ± 166.12	303.15 ± 71.8	504.9 ± 19.37
	All Males	715.15 ± 175.24 #	298.55 ± 96.69 #	518 ± 82.22 #
Female	NS	458.43 ± 13.85	222.27 ± 76.17	314.3 ± 52.27
	MTS	426.25 ± 19.02	146.7 ± 61.8	325.85 ± 16.76
	CTL	514.07 ± 37.42	274.52 ± 97.84	388.83 ± 70.08
	All Females	466.25 ± 23.43	214.5 ± 78.6	342.99 ± 46.61

NS = Neonatal Stress, MTS = Mechanical Tactile Stimulation, CTL = Control; # $p<0.05$ vs. All Females

Abdominal VAT depot relative to abdominal SAT depot

Evaluation of abdominal adipose depot allocation showed that MTS decreased the VAT to SAT ratio in females compared to Neonatal Stress (ANOVA, p=0.02) and Control (ANOVA, p=0.03) groups (Figure 1). No differences were detected in adult males across treatment groups.

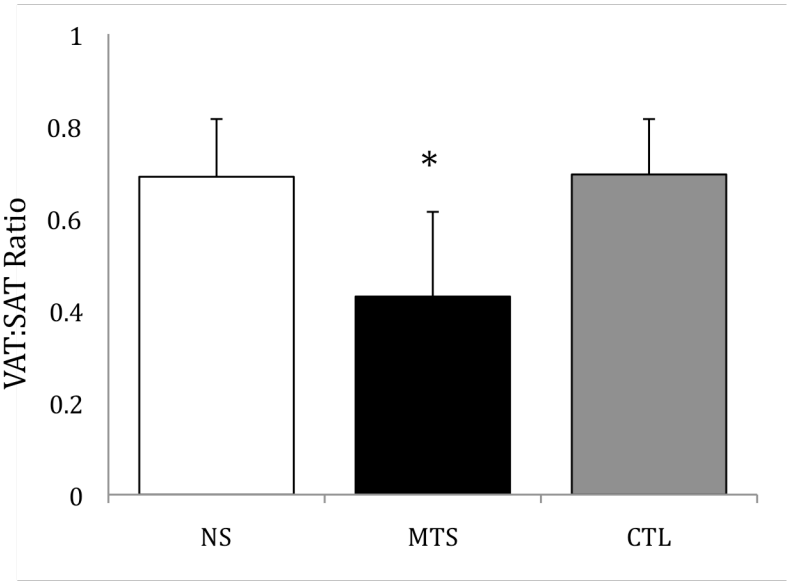

Figure 1: MTS reduced the overall VAT:SAT in adult females. Neonatal Stress is represented by the white bar, MTS by the black bar, and Control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05.

Hyperinsulinemia

MTS during neonatal stress was associated with normal fasting insulin levels in adult males compared to Neonatal Stress alone (ANOVA, p=0.04). See Figure 2. No significant differences in fasting insulin were seen in the females across the three treatment groups. In general, there were significant differences (ANOVA, p=0.02) between the fasting insulin levels of the males compared to females across all treatment groups, with males having higher average circulating insulin than females. Blood glucose levels were found to be similar across treatment and gender and were within normal expected range for adult rats, independent of insulin levels. Data are summarized in Table 3.

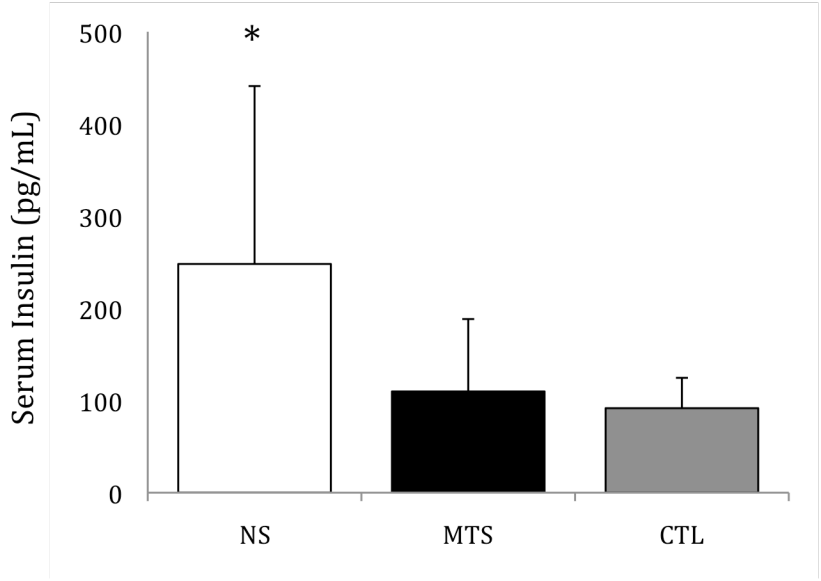

Figure 2: Neonatal stress increased adult male fasting insulin levels. The y-axis represents serum insulin in pg/ml. Neonatal stress is represented by the white bar, MTS by the black bar, and Control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05.

Table 3. Serum fasting blood insulin and blood glucose levels by group and gender. Data is displayed as mean ± standard deviation.

Gender	Group	Insulin	Blood Glucose
Male	NS	248.08 ± 192.99 *	135.5 ± 26.14
	MTS	109.82 ± 78.51	164.86 ± 74.74
	CTL	91.66 ± 32.9	162.75 ± 51.25
	All Males	149.85 ± 101.47 #	154.37 ± 50.81 #
Female	NS	63.3 ± 35.46	172.57 ± 84.93
	MTS	93.26 ± 74.37	123.2 ± 8.98
	CTL	67.86 ± 43.13	117.14 ± 20.13
	All Females	74.8 ± 50.97	137.63 ± 38.01

NS = Neonatal Stress, MTS = Mechanical Tactile Stimulation, CTL = Control; * $p<0.05$ vs. MTS and CTL
#$p<0.05$ vs. females

IL-6 mRNA and protein expression

Neonatal stress significantly decreased the expression of VAT depot IL-6 mRNA in male MTS and Neonatal Stress groups (ANOVA, p=0.05). See Figures3. In adult females, Neonatal Stress had increased IL-6 mRNA expression compared to MTS and Control groups. See Figure 4. There were no significant differences found in SAT depot mRNA expression between any of the treatment groups or genders.

The SAT depot IL-6 protein expression was significantly decreased in both MTS and neonatal stress groups compared to controls (ANOVA, p=0.001). See Figures 5 and 6. This effect was seen in both genders. There were no significant differences in VAT depot IL-6 protein expression. A post hoc test of VAT IL-6

expression using least squares means showed significantly increased expression in Neonatal Stress females compared to Control females (p=0.03) or MTS females (p=0.005). Circulating IL-6 was undetectable across all samples. Data are summarized in Table 4.

VAT TNF-α mRNA and protein expression

MTS during neonatal stress resulted in decreased amounts of adult VAT TNF-α mRNA expression compared to neonatal stress and control groups across both

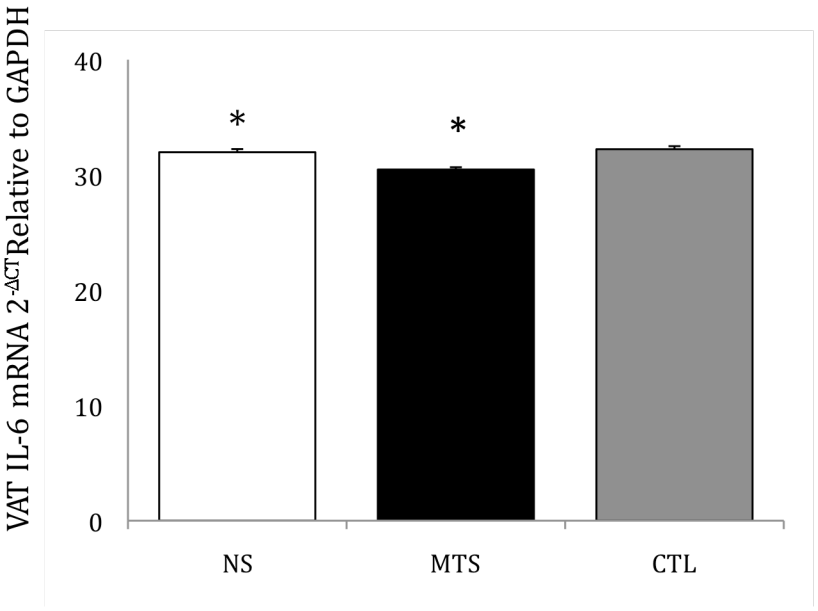

Figure 3: Neonatal stress decreased VAT IL-6 mRNA expression in males compared to controls. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05 vs. CTL

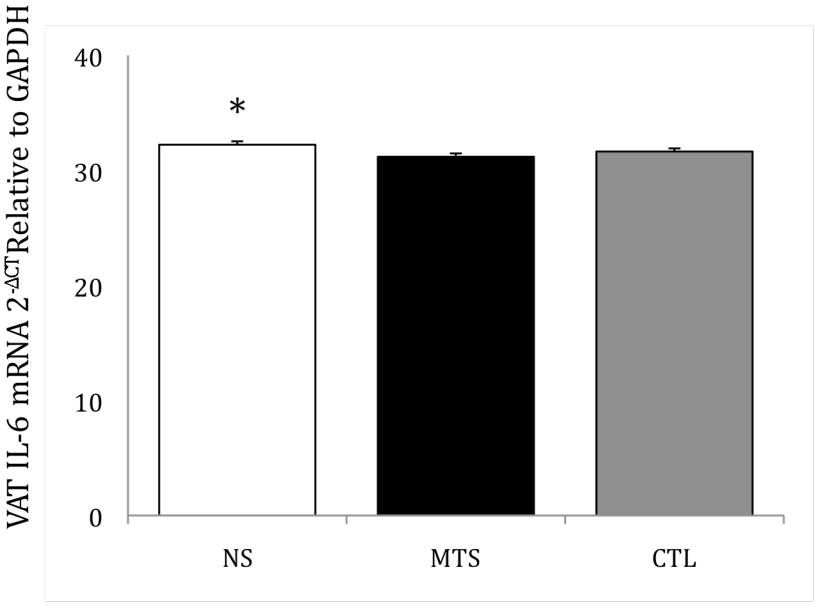

Figure 4: NS had increased VAT IL-6 mRNA expression in females compared to controls. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *$p<0.05$

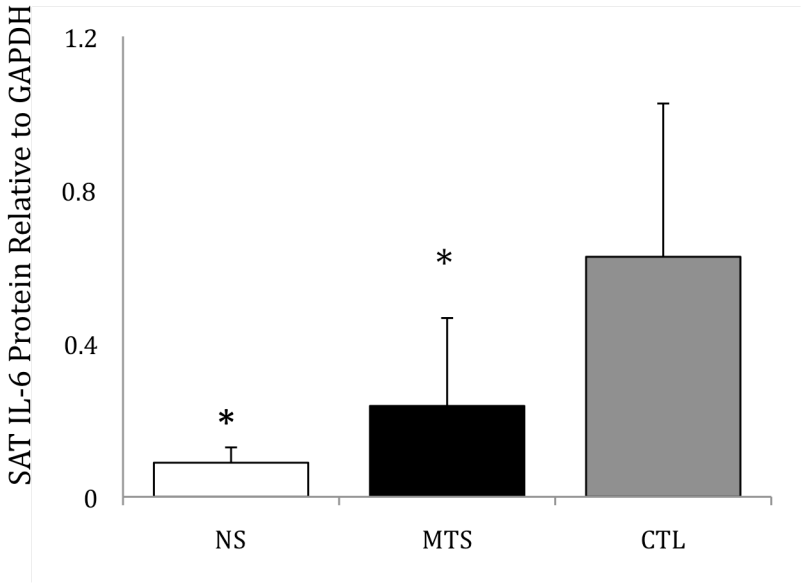

Figure 5: Neonatal stress decreased SAT IL-6 protein expression in males compared to controls. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05.

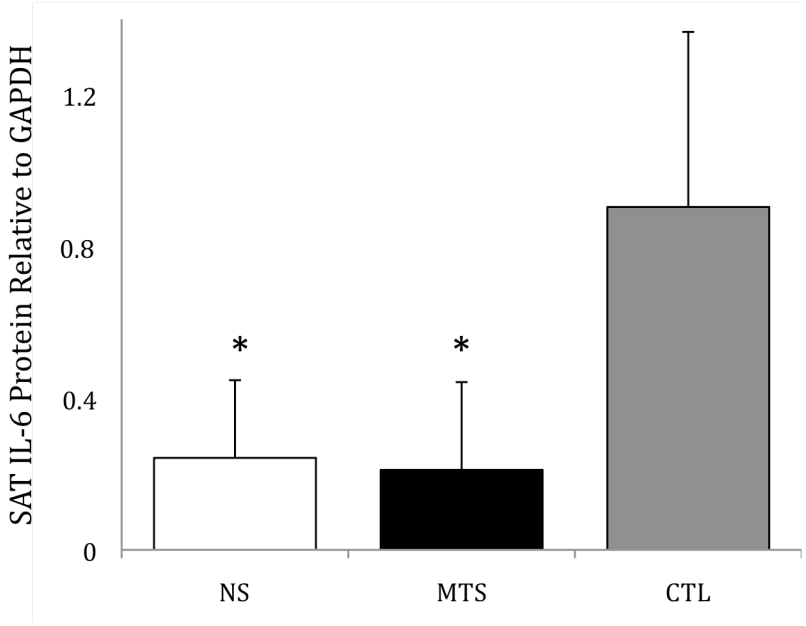

Figure 6: Neonatal stress decreased SAT IL-6 protein expression in females compared to controls. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05.

Table 4. IL-6 mRNA and protein expression in VAT and SAT. Data is displayed as mean ± standard deviation.

Gender	Group	VAT IL-6 mRNA	SAT IL-6 mRNA	VAT IL-6 Protein	SAT IL-6 Protein
Male	NS	31.97 ± 0.27 *	32.33 ± 0.31	0.34 ± 0.29	0.09 ± 0.04 *
	MTS	30.47 ± 0.20 *	30.71 ± 0.29	0.29 ± 0.21	0.24 ± 0.23 *
	CTL	32.23 ± 0.26	31.16 ± 0.32	0.45 ± 0.27	0.63 ± 0.27
	All Males	31.56 ± 0.24	31.4 ± 0.3	0.36 ± 0.26	0.32 ± 0.18
Female	NS	31.64 ± 0.27 *	32.95 ± 0.32	0.56 ± 0.35	0.24 ± 0.21 *
	MTS	31.18 ± 0.29 *	31.44 ± 0.29	0.03 ± 0.01	0.21 ± 0.23 *
	CTL	32.37 ± 0.29	31.07 ± 0.32	0.17 ± 0.067	0.91 ± 0.46
	All Females	31.73 ± 0.28	31.82 ± 0.31	0.25 ± 0.14	0.21 ± 0.33

NS = Neonatal Stress, MTS = Mechanical Tactile Stimulation, CTL = Control; *p<0.05 vs. CTL

genders (ANOVA, p=0.05). See Figures 7 and 8.

Protein expression of TNF-α was not different among groups or genders in either SAT or VAT. However, there was a trend for decreased TNF-α expressed in SAT in the MTS males compared to Neonatal Stress and Controls (ANOVA, p = 0.08). Circulating TNF-α was not detectable across all samples. Data are summarized in Table 5.

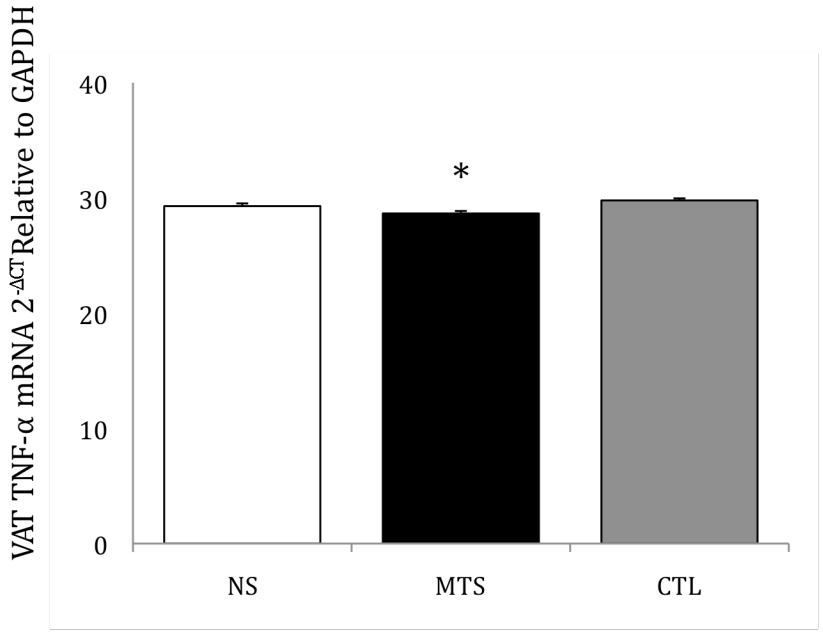

Figure 7: MTS decreased VAT TNF-α mRNA expression in male adult rats. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05

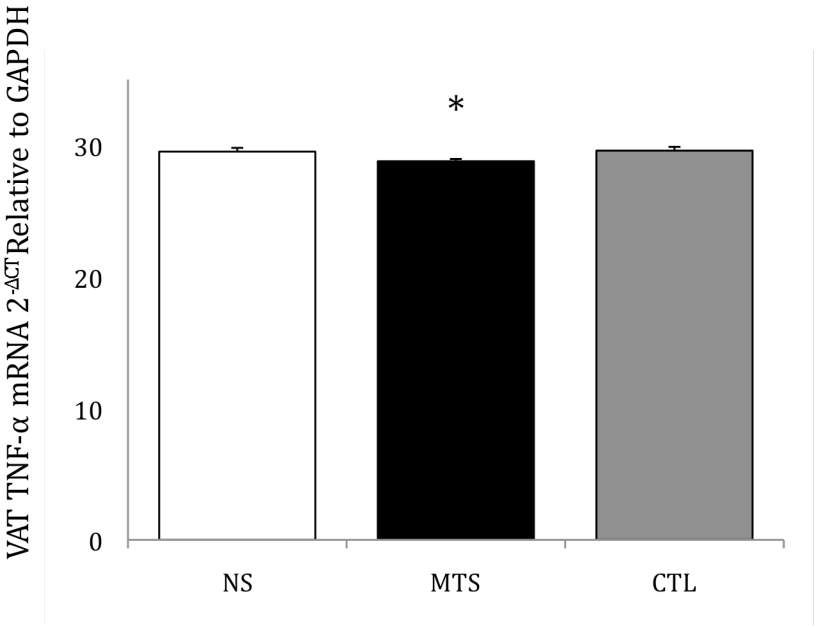

Figure 8: MTS decreased VAT TNF-α mRNA expression in female adult rats. Neonatal stress is represented by the white bar, MTS by the black bar, and control by the grey bar. Data presented as the estimated mean ± standard deviation. ANOVA for treatment with gender as co-factor. *p<0.05.

Table 5. TNF-α mRNA and protein expression in VAT and SAT. Data are displayed as mean ± standard deviation.

Gender	Group	VAT TNF-α mRNA	SAT TNF-α mRNA	VAT TNF-α Protein	SAT TNF-α Protein
Male	NS	29.29 ± 0.24	31.59 ± 0.24	0.96 ± 0.25	1.34 ± 0.25
	MTS	28.65 ± 0.21 *^	31.22 ± 0.32	0.8 ± 0.54	0.17 ± 0.21
	CTL	29.78 ± 0.18	31.01 ± 0.24	1.47 ± 1.69	1.31 ± 1.58
	All Males	29.24 ± 0.21	31.27 ± 0.27	1.08 ± 0.83	0.94 ± 1.01
Female	NS	29.52 ± 0.28	32.32 ± 0.26	0.62 ± 0.22	0.98 ± 0.31
	MTS	28.78 ± 0.17 *^	30.89 ± 0.32	0.31 ± 0.22	1.06 ± 0.83
	CTL	29.59 ± 0.29	30.32 ± 0.32	0.62 ± 0.77	1.45 ± 0.57
	All Females	29.2 ± 0.25	31.18 ± 0.3	0.51 ± 0.40	1.17 ± 0.57

NS = Neonatal Stress, MTS = Mechanical Tactile Stimulation, CTL = Control; * p<0.05 vs. NS, ^ p< 0.05 vs. CTL

DISCUSSION

Utilizing an animal model designed to simulate the stress experienced by neonates in the newborn intensive care unit, we identified specific, MTS-modulated changes in subcutaneous and visceral adipose tissue distribution, fasting insulin levels, and markers of inflammation in adult rats. These findings are significant because MTS, as a means of attenuating the neonatal stress response, may decrease the metabolic consequences in adulthood.

Our findings suggest that early-life stress can have lasting effects on body fat deposition and expression of adipose tissue pro-inflammatory cytokines into adulthood, independent of obesity. It is know that stress increases circulating glucocorticoids which then lead to hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis. This hyperactivity then causes dysregulation of insulin-like growth factor – 1 (IGF-1) expression and activity, resulting in retarded lean tissue growth and increased VAT and SAT deposition. Additional adipose tissue increases lipolytic activity, glucocorticoid and cytokine production with further hyperactivation of the HPA axis. Excessive endogenous and exogenous glucocorticoid exposure is linked to obesity with increased abdominal adiposity, which, in turn, contribute to insulin resistance and the development of metabolic syndrome [11,23], independent of body weight. MTS is hypothesized to attenuate the stress response and reduce adipose tissue deposition through promoting proper

HPA axis function and maturation. Because of the positive feedback relationship between stress and adipose tissue, we expected to see increased body fat in the groups that experienced additional stress, MTS and NS, compared to controls. Based on this premise, we hypothesized that MTS treatment would result in a decrease in adipose tissue even beyond that seen in controls. The animals in the current study had great amounts of body fat, however, based on body weight, they would not be considered obese. There were no differences detected in body weight or body composition, including total body fat, between the three groups (NS, MTS, CTL) at D120. A study by Guzzetta et al [18] looked at the effects of massage compared to a maternal separation animal model. They used three groups of neonatal Long-Evans Hooded rats – maternal separation group which experienced 10 minutes of maternal separation, massage group which received 10 minutes of MTS during the maternal separation period, and a control group that did not experience any maternal separation or massage. At D12, they found that body weight was higher in the maternal separation and MTS groups compared to controls, and that between the two, the maternal separation group weight was higher than that of the MTS pups ($p<0.05$), although this difference disappeared by D25. Guzzetta did not measure body fat percentage, but given our findings we speculate that the maternal separation group had more body fat than either the MTS or control groups. Chen et al [24] looked at the effects of massage therapy from D6 to D10 of life in weanling (D21) and adolescent (D60) rats (n=48) compared to a control group receiving no massage therapy. They found that at D21, animals that received massage therapy were heavier than controls with greater lean body mass. Again, these differences

did not persist through D60 as there was no difference in body weight or fat mass detected. Similarly, we did not find significant differences in VAT and SAT depots across groups and genders at D120. Interestingly, significant differences were detected for the VAT:SAT ratio, with MTS having the lowest compared to NS and CTL groups,. However, it is not clear why these results were not reflected in the separate VAT and SAT depot measures. It is known, however, that having a high VAT:SAT ratio increases the risk of metabolic disease.

Markers of metabolic disease go beyond body weight and fat mass. Fasting glucose and insulin levels are associated with body weight are used today to determine risk for development of type 2 diabetes mellitus. In the present study, fasting blood glucose was similar across treatment or genders at D120; however MTS prevented hyperinsulinemia in males compared to NS animals. Our results observed in NS rats are similar to the findings of McPherson et al [25] who showed hyperglycemia in addition to increased insulin resistance in both male (n=19) and female (n=24) rodents exposed to hypoxia-induced neonatal stress ($p<0.05$). Additionally, Moyer-Mileur et al [20] reported metabolic changes associated with MTS treated neonatal-stressed rats at weaning (D21). Using the same neonatal stress model these investigators found significant improvements in body composition and serum glucose and insulin in animals that received MTS compared to the stress group ($p<0.05$). Specifically, hyperinsulinemia was detected in neonatal stress pups while those that also received MTS had normal fasting insulin levels. We speculate that the decreased circulating insulin levels in our MTS animals are a result of improved insulin signaling and tissue sensitivity.

VAT, as opposed to SAT, preferentially secretes proinflammatory cytokines. Other rodent models of stress have shown the negative impact of stress on proinflammatory cytokine expression [26,27] and adipose tissue depot dynamics. Because of this relationship, we expected to see increases in circulating and adipose tissue-derived TNF-α and IL-6 from the animals with the most visceral fat, in this case, neonatal stress animals. We found that IL-6 mRNA expression was increased in visceral fat in the NS males and females compared to MTS and CTL groups. Additionally, MTS group showed decreased visceral TNF-α mRNA and IL-6 protein expression across both genders. Interestingly, we did find that Control animals often had similar proinflammatory cytokine expression to the NS and MTS groups. Studies have shown that maternal separation alone can induce permanent, negative alterations in neonatal health [27-29], which may account for the elevated cytokines seen in Controls.

IL-6 mRNA expression differences were observed between controls compared to NS and MTS groups. This was surprising because of the complementary and closely linked roles of TNF-α and IL-6 [6,23,30]. Conversely, Mohamed-Ali et al [6] reported an excessive amount of fat (mean body mass index = 31.6) and plasma IL-6 concentrations that correlated with both the VAT:SAT ratio (waist:hip ratio) and percent body fat ($n=39$, $p<0.001$) in overweight men and women. They did not detect differences in TNF-α levels. In mice, Nieto-Vasquez et al [31] showed that prolonged IL-6 exposure resulted in insulin resistance and hyperglycemia. It is possible that, had we seen greater differences in IL-6,

particularly circulating IL-6, we would have detected synchronic differences in glucose and insulin as well.

Stressful events, particularly in early life, establish a fat deposition pattern preferentially to VAT and thus result in increased circulating levels of proinflammatory cytokines. Infant MTS modulates the stress response of hospitalized, preterm infants, possibly reducing their risk of developing metabolic disease. MTS with our neonatal stress model provides insight into the reduction of the stress response through MTS therapy in preterm infants and the possible reversal of some of the negative effects of early-life stress.

There are several factors that limit this study. The neonatal animal model of stress provides us with the best method of understanding the effects of MTS treatment in vivo, however, inherent differences between animals and humans pose some unavoidable limitations. It is impossible for an animal model to mimic the human condition exactly, however, the maximum amount of control was used to reduce any extraneous variable differences that may have compromised the data. Additionally, findings from animal studies regarding fat deposition and insulin resistance correlate with recent clinical findings in preterm adults. Another limitation was our inability to achieve detectable serum protein levels of TNF-α and IL-6, although this is not an uncommon barrier with these cytokines. Using ultrasensitive ELISA kits may have resulted in attaining detectable values of TNF-α and IL-6. Lastly, this study does not include a naïve control group for baseline comparison.

Despite these limitations and in conclusion, this study substantiates the notion that neonatal stress can have a negative impact on body composition, cytokine expression and development of metabolic syndrome. Additionally, it shows that MTS administered during neonatal stress leads to altered adipose tissue deposition, reduced hyperinsulinemic response, and changes in depot-specific expression of proinflammatory cytokines, TNF-α and IL-6, independent of body weight. This suggests that MTS may be a viable intervention in attenuating the negative effects of early-life stress among neonatal stressed rats.

REFERENCES

1. Tamashiro KLK, et al. Social stress and recovery: Implications for body weight and body composition. *Am J Physiol Regul Integr Comp Physiol* 2007; 293:1864—1874.

2. Charmandari E, Tomoshige K, Souvatzoglou E, Chrousos. Pediatric stress: Hormonal mediators and human development. *Horm. Res.* 2003; 59:161—179.

3. Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiol. Behav.* 2008; 94:206—218.

4. Fantuzzi G, Adipose tissue, adipokines, and inflammation. *J Allergy Clin. Immunol.* 2005; 115(5): 911—919.

5. Shi H, Seeley RJ, and Clegg DJ. Sexual differences in the control of energy homeostasis. *Frontiers in Neuroendocrinology* 2009; 30:396—404.

6. Mohamed-Ali V, et al. Subcutaneous adipose tissue releases interluekin-6, but not tumor necrosis factor-α, *in vivo*. *J. Clin. Endocrinol. Metab.* 1997; 82(12):4196—4200.

7. Kim JK. Inflammation and insulin resistance: An old story with new ideas. *Korean Diabetes Journal* 2010; 34: 137—145.

8. Tappy L, Alessio DD. Obesity and insulin resistance: Is it due to body fat, energy balance, or gut factors? *Cur. Opin. Clin. Nutr. Metab. Care* 2006; 9:455—457.

9. Samaras K, Botelho NK, Chisholm DJ, Lord RV. Subcutaneous and visceral adipose tissue gene expression of serum adipokines that predict type 2 diabetes. *Obesity* 2009; 443:1—6.

10. Glukman, PD. Editorial: Nutrition, glucocorticoids, birth size, and adult disease. *Endocrinology* 2001; 142(5):1689—1691.

11. Seckl JR. Glucocorticoid programming of the fetus; Adult phenotypes and molecular mechanisms. *Mol. Cell. Endocrinol.* 2001; 185:61—71.

12. Uthaya S, Thomas EL, Hamilton G, Doré CJ, Bell J, Modi N. Altered adiposity after extremely preterm birth. *Pediatr Res* 2005 Feb;57(2):211—215.

13. Ong KK. Size at birth, postnatal growth and risk of obesity. *Horm. Res.* 2006; 65(3):65—69.

14. Weaver ICG, Meany MJ, Szyf M. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *PNAS* 2006; 103(9):3480—3485.

15. Hovi P, Anderson S, Eriksson JG, Javenpaa A, Stranng-Karlsson S, Makitie O, and Kajantie E. Glucose regulation in young adults with very low birth weight. *N Engl J Med* 2007; 356:2051—63.

16. Massaro AN, Hammand TA, Jazzo B, Aly H. Massage with kinesthetic stimulation improves weight gain in preterm infants. *Am. J. Perinatol.* 2009; 29:352 – 357.

17. Diego MA, Field T, Hernandez-Reif M. Procedural pain heart rate responses in massaged preterm infants. *Infant Behav Dev.* 2009; 32(2):226—229.

18. Guzzetta A, et al. Massage accelerates brain development and the maturation of visual function. *J. Neurosci*. 2009; 29(18):6042—6051.

19. Weber BC, Manfredo HN, Rinaman L. A potential gastrointestinal link between enhanced postnatal maternal care and reduced anxiety-like behavior in adolescent rats. *JBNR*. 2009; 123(6):1178—1184.

20. Moyer-Mileur, LJ. Mechanical-tactile stimulation (MTS) during neonatal stress prevents hyperinsulinemia despite stress-induced adiposity in weanling rat pups. *Early Human Development*. 2011; 87(3):159 – 63.

21. Ferreira AM, Bergamasco NHP. Behavioral analysis of preterm neonates included in a tactile and kinesthetic stimulation program during hospitalization. *Rev Bras Fisioter.* 2010; 14(2)141—148.

22. Field T, et al. Insulin and insulin-like growth factor 1 (IGF-1) increased in preterm infants. *J Dev Behav Pediatr.* 2008; 29(6):463—466.

23. Crousos GP, Tomoshige K. Glucocorticoid signaling in the cell: Expanding clinical implications to complex human and behavioral somatic disorders. *Ann N Y Acad Sci* 2009; 1179:153—156.

24. Chen H, et al. Massage therapy during early postnatal life promotes greater lean mass and bone growth, mineralization, and strength in juvenile and young adult rats. *J. Musculoskelet. Neuron. Interact.* 2009; 9(4):278 – 287.

25. McPherson RJ, Mascher-Denen M, Juul SE. Postnatal stress produces hyperglycemia in adult rats exposed to hypoxia-ischemia. *Pediatric Research.* 2009; 66(3):278—282.

26. Coppack SW. Pro-inflammatory cytokines and adipose tissue. *Proceedings of the Nutrition Society* 2001; 60: 349—356.

27. Gareau MG, Wine E, Sherman PM. Early life stress induces both acute and chronic colonic barrier dysfunction. *NeoReviews*. 2009;10:191 – 197.

28. Coutino SV, et al. Neonatal maternal separation alters stress-induced responses to viscersomatic nociceptive stimuli in rat. *Am. J. Physiol Gastrointest. Liver Physiol.* 2002;282:307 – 316.

29. Francis DD, et al. Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J. Neurosci*. 2002; 22(18):7840 – 7843.

30. Kyrou I, Chrousos GP, Tsigos C. Stress, visceral obesity and metabolic complications. *Ann. N Y Acad. Sci.* 2006; 1083:77—110.

31. Nieto-Vasquez I, et al. Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. *Diabetes.* 2008; 57(12): 3211–3221.