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COMPARISON OF DIET VS. EXERCISE ON METABOLIC FUNCTION & GUT MICROBIOTA IN OBESE RATS

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Abstract

Cardiometabolic impairments that begin early in life are particularly critical, as they often predict metabolic dysfunction into adulthood. Obesity, high-fat diet (HFD), and inactivity are all associated with adipose tissue (AT) inflammation and insulin resistance (IR), major predictors of metabolic dysfunction. Recent evidence also has associated the gut microbiome with cardiometabolic health.

PURPOSE—Compare equal energy deficits induced by exercise and caloric reduction on cardiometabolic disease risk parameters including AT inflammation, IR and gut microbiota changes during HFD consumption.

METHODS—Obesity-prone rats fed HFD were exercise trained (Ex, n=10) or weight-matched to Ex via caloric reduction while kept sedentary (WM, n=10), and compared to *ad libitum* HFD-fed (Sed, n=10) rats for IR, systemic energetics and spontaneous physical activity (SPA), adiposity, and fasting metabolic parameters. Visceral, subcutaneous, periaortic, and brown AT, liver, aorta, and cecal digesta were examined.

RESULTS—Despite identical reductions in adiposity, Ex, but not WM, improved IR, increased SPA by ~26% (p<0.05 compared to WM and Sed), and reduced LDL cholesterol (p<0.05 compared to Sed). WM and Ex both reduced inflammatory markers in all AT depots and aorta, while only Ex increased indicators of mitochondrial function in brown AT. Ex significantly increased the relative abundance of cecal *Streptococcaceae* and decreased *S24-7* and one undefined genus in *Rikenellaceae*; WM induced similar changes that did not reach statistical significance.

CONCLUSIONS—Both Ex and WM reduced AT inflammation across depots, while Ex caused more robust changes to gut microbial communities, improved IR, increased fat oxidation, increased SPA, and increased indices of brown AT mitochondrial function. Our findings add to the

growing body of literature indicating that there are weight-loss independent metabolic benefits of exercise.

Keywords

adiposity; energy expenditure; brown adipose tissue; insulin resistance; spontaneous physical activity; gut microbiome

INTRODUCTION

For the first time in history, children are expected to have a decline in life expectancy, which is largely due to the increased prevalence of obesity. The health implications of excess adiposity early in life may be especially impactful, as this is a critical period of development. Similar to adults, obese and overweight children present with metabolic perturbations such as insulin resistance (IR), and progress to develop Type 2 diabetes and coronary artery disease (11). While the root cause of obesity is multifactorial involving biological, genetic and environmental factors, the key elements are excessive intake of energy-dense foods and physical inactivity. Recent controversy exists over whether reduced total caloric intake (i.e., “diet”) may be a more efficacious approach than exercise to reduce excess body weight (33), although the efficacy of diet versus exercise for weight loss has not been adequately tested, especially in children. Importantly, there is increasing evidence that implicates a habitually sedentary lifestyle as a major independent predictor of disease (4); accordingly, de-incentivizing exercise may have dire public health consequences, especially among children who develop physical activity habits early and who will experience cumulative effects of sedentarism by the time they reach adulthood. Because any energy deficit results in weight loss, it is difficult to ascertain information on the weight loss-independent effects of exercise and diet. It is important to determine if exercise reduces cardiometabolic disease risk factors independent of weight loss in order to support public health campaigns to encourage physical activity among adolescents, especially in light of mixed messages in the media regarding the importance of physical activity for weight loss.

The mechanisms linking obesity to its associated cardiometabolic diseases have not been fully elucidated; however, adipose tissue (AT) inflammation is known to play a central role (27). Meanwhile, physical activity improves metabolic health even when weight loss is not achieved by enhancing insulin sensitivity; and increasing evidence demonstrates an exercise-mediated decrease in white AT (WAT) inflammation in rodents and humans. In addition, new evidence shows that exercise may initiate WAT ‘browning’ indicated by increased multilocular lipid droplet accumulation and mitochondrial density (12). As WAT adopts a “brown” or “beige” phenotype, the increased mitochondrial uncoupling (i.e., increased expression of mitochondrial uncoupling protein (UCP-1)) increases its thermogenic capacity. Moreover, beige AT has been demonstrated to be metabolically healthier than WAT and associates with greater whole body insulin sensitivity; similar findings link brown AT (BAT) activity with improved systemic metabolic health (2). To date, no studies have comprehensively investigated the energy deficit-equivalent effects of voluntary exercise versus diet on AT immunometabolic characteristics across depots, in addition to systemic cardiometabolic effects and changes to the gut microbiome. Thus, the purpose of this study

was to make those comparisons using an animal model of juvenile obesity development in which the energy deficit due to exercise versus diet could be carefully matched. Since BAT content decreases with age, we deemed it important to examine these effects on BAT at this early developmental stage, theoretically characterized by having greatest BAT content.

Strong associations have been made between the composition of gut microbial communities and metabolic abnormalities related to obesity in humans (22). Remarkably, obesity can be induced in a host organism (i.e., lean gnotobiotic mice) when exposed to gut microbiota from obese donor mice (34). Shifts in gut microbial communities have therefore been proposed to contribute to the development of obesity. Whether weight loss, either through exercise or calorie reduction, can shift gut microbial communities, specifically during the progression of early life obesity, is completely unknown, yet recent work highlights the importance of elucidating relationships between exercise and gut health in humans (9). A better understanding of how these factors influence the gut at this critical period may offer insight into how they may augment susceptibility to obesity and related metabolic conditions later in life. We recently demonstrated that exercise impacts the composition of gut microbial communities in adult rats with low aerobic capacity (25). Whether those changes were due to the exercise training *per se*, or the energy deficit caused by exercise, was not addressed. These questions are difficult to answer in humans, where it is difficult to carefully control energy intake and expenditure. Thus, a well-controlled study involving an animal model is useful in this regard. Moreover, use of an animal model allows for the analysis of relationships between tightly controlled behavioral variables (e.g., diet and exercise) and tissue-specific changes that would be not be possible in humans due to their invasive nature.

The obesity-prone rat is known to develop diet-induced obesity, IR, hyperinsulinemia and hypertriglyceridemia. Because exposure to an HFD is an independent contributor to metabolic dysfunction, we controlled for this by feeding all rats a typical 'Western' diet high in fat and sucrose. The exercise training protocol was voluntary wheel running. We created a weight-matched sedentary control group by limit-feeding, in order to match the energy deficit induced by voluntary wheel running in the exercise group. In this way, we were able to investigate the independent metabolic benefits of both weight gain attenuation via caloric deficit and exercise. We tested the hypotheses that, when matched for changes in adiposity, voluntary exercise (compared to diet) would result in improved cardiometabolic outcomes including enhanced insulin sensitivity, increased fat oxidation, and improved immunometabolic parameters across four AT depots. Additionally, we aimed to explore whether exercise and an equivocal dietary energy deficit differ in their ability to initiate browning of WAT and metabolic activity of BAT. Finally, we determined whether differences exist in how exercise and diet shift gut microbial communities, and whether any such differences relate to metabolic differences resulting from exercise versus dietary energy deficit.

MATERIALS AND METHODS

Animals and Diets

Thirty male, obesity-prone (OP-CD) rats were obtained at 4 weeks of age (Charles River Laboratories, Inc., O'Fallon, MO) and housed individually at the Animal Sciences Research

Center at the University of Missouri-Columbia. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri. Animal quarters were temperature-controlled at 21°C with alternating 12-h light:dark cycles from 0800 to 2000 and 2000 to 0800, respectively. Immediately upon arrival, rats were randomized to one of the following three groups: 1) HFD, sedentary (“Sed;” $n = 10$), 2) HFD, exercise via voluntary wheel running (“Ex;” $n = 10$), or 3) HFD, weight-matched to Ex (“WM;” $n = 10$). HFD (D12451; Research Diets, Inc., New Brunswick, NJ) contained ~20% protein, 35% carbohydrate, and 45% kcal from fat with the predominant fatty acid type being saturated derived from lard. Animals in the Sed and Ex groups were fed *ad libitum* for the duration of the study. The “Ex” group were housed with running wheels (Tecniplast Activity Wheel; West Chester, PA) connected to a bicycle pedometer (Sigma BC 509; Sigma, St. Charles, IL) to record weekly running distance. Animals in the WM group were provided with 75-80% of the food consumed by the Sed group, based on daily body weight comparisons to ensure weight-matching to the Ex group. Food intake was recorded daily for the WM and Sed groups while that of the Ex group was recorded weekly. Rats were anesthetized via intraperitoneal (IP) injection of pentobarbital sodium (50mg/kg) between 18-20 weeks of age and then euthanized by exsanguination via cardiac puncture before tissues were harvested.

Energy Expenditure and Spontaneous Physical Activity (SPA)

At 11 weeks of age, animals ($n = 8$ /group) were placed in metabolic chambers (Promethion; Sable Systems International, Las Vegas, NV) to assess total energy expenditure (TEE), resting energy expenditure (REE), and respiratory quotient (RQ) via indirect calorimetry, and SPA by the summation of x-, y-, and z-axis beam breaks. Body weight and food intake were measured before and after each 48-h assessment. Each 48-h run captured at least two light and two dark cycles. During this time, wheels were removed from the Ex animals and food was administered *ad libitum* to the WM animals. Although both treatments were temporarily removed, the duration of the metabolic assessment was short to avoid any effects of de-training in the Ex group or disruption of weight-matching in the WM group.

Insulin Resistance

Insulin resistance (IR) was measured indirectly via IP glucose tolerance tests (GTT) at 12 weeks of age. Animals were fasted overnight prior to receiving assessment the following morning at 0800. Following the collection of fasting glucose measurements, an IP injection of 50% dextrose (2g/kg) was administered. Blood samples (<200 μ L) were collected at 15, 30, 45, 60 and 120 minutes following injection from the lateral tail vein via venipuncture to measure glucose concentration (AlphaTRAK, Abbott Animal Health; Chicago, IL). After collection, samples were centrifuged at 4°C for 10 minutes at 10,000 g and plasma aliquots were stored at -80°C for further analysis. Using fasting glucose and insulin values, Homeostatic Assessment of Insulin Resistance (HOMA-IR) was calculated and using glucose and insulin measures throughout the GTT, the Matsuda Index (MI) was calculated.

Circulating Metabolic Markers

On the day of sacrifice, fasting blood was collected for hemoglobin A1c (HbA1c) assessment in whole blood, and plasma concentrations of several metabolic parameters.

Plasma samples were stored at -80°C until further analysis for glucose, insulin, cholesterol, triglycerides, high- (HDL) and low-density lipoproteins (LDL) as well as non-esterified fatty acids (NEFA) using commercially available assays, according to manufacturer's guidelines, by a third party laboratory (Comparative Clinical Pathology Services; Columbia, MO).

Tissue Harvest

Liver, aorta, and AT from inguinal subcutaneous (SQAT), visceral epididymal (EpiAT), visceral retroperitoneal (RPAT), visceral omental (OMAT), periaortic (PAT) and interscapular BAT depots were harvested, weighed, and either stored immediately at -80°C until further analysis or fixed in 10% formalin for histological assessment. Cecal digesta was collected and stored at -80°C for microbial analysis. Samples were immediately flash frozen in liquid nitrogen then stored at -80°C until further analysis. BAT samples were also allocated for histology fixation.

Histology

Formalin-fixed BAT samples were embedded in paraffin, sectioned at $5\mu\text{m}$, and stained with hematoxylin and eosin (H&E). Sections were examined at 20x magnification for phenotypic assessment of lipid droplets (e.g., multilocularity of brown adipocytes). A third party blindly assessed adipocyte images in order to obtain a representative image for each group.

RNA Extraction and real time qPCR

SQAT, EpiAT, PAT, BAT and aorta samples were homogenized in TRIzol solution using a tissue bead homogenizer (TissueLyzer, Qiagen; Valencia, CA). mRNA was extracted according to Qiagen's RNeasy lipid tissue protocol and assessed for purity (260/280) and concentration ($\text{ng}/\mu\text{L}$) using a NanoDrop 1000 (NanoDrop Technologies, Inc; Wilmington, DE). cDNA was reverse transcribed from total mRNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). PCR was performed as previously described (15). All mRNA data represented utilized the C_T approach, comparing gene expression relative to the housekeeping genes, 18S and glyceraldehyde 3 phosphate dehydrogenase (GAPDH); data are expressed as fold difference relative to Sed.

Liver Triglycerides

Hepatic triacylglycerol (TG) concentration was determined using a commercially available kit (Wako L-Type TG M, Wako Pure Chemical Industries, Ltd.; Osaka, Japan) as previously described (36). Data are expressed as milligrams TG per wet weight liver.

Citrate Synthase Assay

Citrate synthase activity was determined following previously described methods by Srere et al. (32). Briefly, homogenized BAT samples were incubated in solution with oxaloacetate, acetyl-CoA, and DTNB and citrate synthase enzyme activity was determined by spectrophotometric absorption set at 412 nm.

Western Blot

Western blot analysis was performed on protein lysates isolated from tissues as previously described (36). To determine mitochondrial density, determination of oxidative phosphorylation (OxPhos) complexes I through V of the electron transport chain (MitoProfile Total OxPhos Rodent WB Antibody Cocktail, MitoSciences; Eugene, OR) were measured. Mitochondrial content of each complex was determined by the density of each band of the phosphoprotein divided by the density of the total protein. To control for equal protein loading and transfer, the membranes were stained briefly with 1% amido black as previously described (36). Total protein loaded in each lane was quantified and these values were used to adjust for any differences in protein loading or transfer of all band densities. Data are expressed relative to Sed.

Cecal Digesta DNA Extraction, Amplification, Sequencing and Bioinformatics

Total DNA from cecal digesta samples was extracted using Mo-Bio PowerSoil kits (MO BIO Laboratories, Inc., Carlsbad, CA). Concentration of extracted DNA was quantified using a Qubit® 2.0 Fluorometer (Life technologies, Grand Island, NY). 16S rRNA gene amplicons of were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') that target a 291 bp-fragment of V4 region were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (6). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. Quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life technologies, Grand Island, NY) and extracted using Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois. Fluidigm tags were removed using FASTX-Toolkit (version 0.0.13), and QIIME 1.8.0 was used to process the resulting sequence data (5). Briefly, high quality (quality value ≥ 20) sequence data derived from the sequencing process were demultiplexed. Sequences were then clustered into operational taxonomic units (OTU) using closed-reference OTU picking against the Greengenes 13_8 reference OTU database with a 97% similarity threshold. Singletons (OTUs that were observed fewer than 2 times) and OTUs that had less than 0.01% of the total observation were discarded. A total of 804,969 reads were obtained, with an average of 28,748 reads (range = 13,623-99,717) per sample. Rarefaction curves based on observed species, Chao1, and phylogenetic distance whole tree measures clearly plateaued, suggesting sufficient sequencing depth (data not shown). The dataset was rarified to 13,623 OTU for analysis of diversity and species richness. Principal coordinates analysis (PCoA) was performed, using both weighted and unweighted unique fraction metric (UniFrac) distances that measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree, on the 97% OTU composition and abundance matrix (26).

Statistical Analysis

One-way ANOVAs were performed using SPSS v22 to investigate the differences between groups. Post hoc LSD comparisons were utilized if overall model significance between groups was found. Microbial differences (phyla, family, and genera) between groups were analyzed using SAS 9.3 (SAS Institute, Cary, NC) with Mixed Models procedure and post hoc Tukey's tests when appropriate. Pearson correlations between microbiota and the relative abundance of specific taxa were assessed using Qiime 1.9.1, and the p value corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons was used. Statistical significance was concluded when $p < 0.05$. Data are expressed as the mean $\pm$ standard error of the mean (SEM).

RESULTS

Weight-matching resulted in Ex and WM groups having equivocal adiposity

Body weight gain was significantly attenuated with voluntary exercise (i.e., Ex) and, by design, equal to that of the diet (i.e., WM) group, when compared to the sedentary control (i.e., Sed) (**Figure 1A**) resulting in final body weights were ~18% lower ($p < 0.05$) in both intervention groups compared to Sed (Sed, 569 ± 12.89 g; Ex, 467 ± 19.80 g; WM, 476 ± 3.38 g) with no statistical difference between Ex and WM (**Figure 1B**). WM animals had food reduced to ~75% of that consumed daily by the Sed group, allowing them to maintain mean body weights comparable to that of the Ex group for the entire course of the study. Both Ex and WM groups also had reduced WAT mass indicated by lighter SQAT, EpiAT, RPAT, and OMAT depots (**Figure 1C**; all $p < 0.05$ compared to Sed with the exception of OMAT where the difference between Ex and Sed did not reach statistical significance).

Both interventions increased energy expenditure, but only Ex increased fat oxidation

There was no increase in caloric intake in the Ex group fed *ad libitum* (**Figure 1D**). Thus, any differences between the Sed and Ex groups can be attributed to the energy deficit due to exercise, independent of any changes in energy intake. Compared to the Sed, both Ex and WM groups increased TEE, but not necessarily REE, as illustrated in **Figure 1E & H**. The RQ values were ~5-11% lower in the Ex group compared to both Sed and WM groups in both light and dark cycles (all $p < 0.05$), suggestive of greater fat oxidation (**Figure 1G**). Furthermore, within the 48-h metabolic cage assessment, during which time no running wheels were provided, dark (i.e., rodent active) cycle, SPA was significantly increased in the Ex group compared to both Sed and WM groups. SPA was also ~30% higher in both Ex and WM groups compared to Sed during the light (i.e., rodent inactive/sleep) cycle (**Figure 1F**).

Compared to WM, Ex was more effective at improving insulin sensitivity

The Ex, but not the WM group, also had improved insulin sensitivity as assessed via the Matsuda Index (MI) compared to Sed (**Figure 1I**) and HOMA-IR (**Figure 1J**). The greater insulin-sensitivity of the Ex group associated with a reduction in compensatory hyperinsulinemia. In other words, total glucose area under the curve (AUC) during GTT was similarly reduced in both treatment groups, whereas GTT insulin secretion was reduced in

the Ex group. While %HbA1c was reduced in both treatment groups, only the reduction in the WM group reached statistical significance (**Table 1**).

Ex and WM had similar beneficial effects on circulating lipids and ectopic lipid deposition

Total cholesterol was reduced in both treatment groups (24% and 22% reduction compared to Sed for Ex and WM groups, respectively, both $p < 0.05$), while there was no significant reduction in total circulating TG, HDL, or NEFAs in either treatment group. Ex, but not WM, reduced LDL level (Ex versus Sed, $p < 0.05$). As shown in **Table 1**, liver mass was significantly decreased in Ex, but not the WM group. However, while both interventions tended to reduce liver TG content, only the difference between Sed and WM was significant.

Ex and WM affect AT gene expression in a depot-specific manner and both reduce aorta inflammation

Expression levels of a comprehensive panel of genes were measured in two WAT depots (SQAT and EpiAT) as well as BAT, PAT (from the thoracic aorta region), and aorta (see Figure, Supplemental Digital Content 1, Adipose tissue and aorta gene expression). In SQAT, thought to be less susceptible to inflammation, modest changes were observed which were comparable between the two interventions. Although the reductions in both leptin and adiponectin were only significant for the WM group, both Ex and WM were equally effective at reducing the inflammatory macrophage marker, CD11c and DNA-damage inducible transcript-3 (DDIT3), which is indicative of endoplasmic reticulum stress. In visceral WAT, thought to be the depot most susceptible to metabolic inflammation, Ex reduced leptin compared to both Sed and WM groups ($p < 0.05$ for both) and increased the adipokine, adiponectin ($p = 0.021$ compared to Sed), known to be anti-inflammatory and insulin sensitizing, while WM did not alter either of those adipokines. Ex tended to ($p = 0.153$ compared to Sed) increase expression of peroxisome proliferator activated receptor gamma coactivator (PGC1- α), the master regulator of mitochondrial biogenesis. On the other hand, both Ex and WM were comparable in their ability to reduce several markers of inflammation including CD11c, the T lymphocyte markers CD8 and FoxP3, DDIT3, and the p67Phox subunit of NADPH oxidase, an indicator of oxidative stress. Of all of the AT depots investigated, BAT exhibited the greatest gene expression changes. The two interventions both significantly reduced leptin and adiponectin (indicative of reduced “whitening” of BAT), as well as traditional markers of inflammation and T lymphocyte activation (i.e., tumor necrosis factor (TNF)- α , CD8, FoxP3), and markers of ER/oxidative stress (Hspa5, the gene encoding for glucose regulated protein (Grp78) which is an ER chaperone, p22Phox, DDIT3).

Emerging research suggests that the AT surrounding the aorta directly impacts the vasculature (38); however, mechanisms by which exercise training affects this depot in obesity are not known. We assessed gene expression of the AT depot adjacent to (i.e., surrounding) the thoracic aorta, PAT, as well as that of the aorta tissue itself. Surprisingly, the following inflammatory genes were actually up-regulated in PAT of the exercise-trained (but not WM) animals: the chemoattractant molecule, monocyte chemoattractant protein (MCP)-1, the macrophage makers, F4/80 and CD11c, and indicators of ER/oxidative stress (Hspa5 and P67Phox, respectively). On the other hand, WM increased, and Ex decreased,

leptin levels in PAT whereas both interventions reduced adiponectin expression. Inflammatory genes were measured in aorta to determine if the apparent inflammatory effects of exercise on PAT correlated with similar increases in this tissue. Despite divergent effects of both Ex and WM on the surrounding PAT, both weight-loss protocols decreased gene expression of two important proinflammatory cytokines known to be involved in atherogenesis, TNF- α and IL-6.

Ex increases BAT mitochondrial activity, but not uncoupling, and does not induce WAT browning

While BAT mass was not different between the two treatment groups (**Figure 2A**), the ratio of BAT to WAT was significantly increased only in the Ex group compared to Sed (**Figure 2B**). Not only did the Ex group have higher relative BAT mass, but BAT appeared to have greater metabolic activity compared to WM as assessed via protein expression of mitochondrial markers. That is, both citrate synthase activity (**Figure 2C**), and expression of mitochondrial respiratory complexes I and II (**Figure 2D**), were higher in Ex compared to WM ($p < 0.05$). Ex also increased BAT citrate synthase activity by ~11% compared to both the WM and Sed groups (Sed: 2.73 ± 0.05 nmol/min/ug, Ex 3.04 ± 0.09 nmol/min/ug, WM 2.72 ± 0.07 nmol/min/ug, $p < 0.05$ for each). Based on H & E staining, both Ex and WM reduced overall lipid droplet content in BAT compared to the Sed group (**Figure 2H**). However, BAT adipocytes from Ex appeared denser with smaller lipid droplets. WM resulted in an apparently different BAT phenotype characterized by fewer, yet larger, lipid droplets. No mitochondrial genes were modified, except for PGC1- α , whose expression was significantly lowered by WM, and the marker of mitochondrial long chain fatty acid oxidation, carnitine palmitoyltransferase 1b (CPT1b), which was reduced in Ex. The signature uncoupling protein of BAT, UCP-1, was not significantly altered by either intervention. Since recent evidence implicates alternative (i.e., M2) macrophage activity with AT browning (24) and exercise is known to shift macrophages toward M2 (18), the alternative macrophage-polarizing cytokine, IL-13, as well as the M2 macrophage marker, C-type mannose receptor-1 (MRC1), were measured. Interestingly, both genes were found to be higher in the treatment groups, although the diet-induced increase in MRC1 was not statistically significant (**Figure 2F**). No other markers of browning were evident in SQAT (**Figure 2G**).

Ex and WM shift cecal microbial communities

No difference was found between groups in alpha diversity, suggesting that neither Ex nor WM affected species richness in cecal microbiota. However, weighted principal coordinates analysis (PCoA) showed that the beta diversity of gut microbial communities was significantly different ($p = 0.028$) between Sed and Ex groups, as well as between Sed and WM groups, but not between Ex and WM groups (**Figure 3A**). Although the weighted PCoA plot does not appear to have obvious clustering, significance between groups was observed from the UniFrac distance metrics performed and analyzed through non-parametric analysis with Bonferroni corrections. PCoA of unweighted UniFrac distances between samples indicated that gut microbial communities were not different between the three treatment groups (**Figure 3B**). These PCoA results suggested that the abundance of the

bacteria, and not the presence of rare taxa, was the driving force of the differences observed. The major differences between the three groups of rats were as follows.

In total across all three groups, 7 phyla, 33 families, and 54 genera represented usable raw reads. Only a few taxa accounted for the majority at each level. At the phyla level, *Firmicutes* (71.2% of sequences), *Bacteroidetes* (23.0% of sequences), *Verrucomicrobia* (3.6% of sequences) and *Proteobacteria* (1.6% of sequences) represented approximately 99% of the sequences, while the rest of the 3 phyla represented < 1% of total sequences. Neither intervention significantly affected gut microbial community structure at the phylum level; however, Ex and WM both tended to ($p=0.056$) lower the relative abundance of *Bacteroidetes* compared to Sed (see Table, Supplemental Digital Content 2, Bacterial phyla, families and genera of cecal digesta in rats after 8-wk of dietary and exercise intervention). The relative abundance of *Firmicutes:Bacteroidetes* ratio did not differ among treatment groups. On the other hand, Ex significantly increased the relative abundance of the *Streptococcaceae* family, and significantly decreased the relative abundance of an undefined genus in the *Rikenellaceae* family and an undefined genus in the *S24-7* family when compared to Sed. Interestingly, whereas WM tended to decrease the relative abundance of *Streptococcus* compared to Sed, Ex had the opposite tendency; in fact, WM significantly decreased the relative abundance of *Streptococcus* compared to the Ex group. This is especially noteworthy since the Sed and Ex groups consumed exactly the same amount of HFD.

Correlations between a host of metabolic variables and identified taxa identified that the relative abundance of SMB53 ($r=0.6769$, $p=0.002$), an undefined genus in *Peptostreptococcaceae* family ($r=0.6082$, $p=0.011$), and an undefined genus in *Rikenellaceae* family ($r=0.5780$, $p=0.018$) were all positively correlated with total adiposity. The relative abundance of the undefined genus in *Rikenellaceae* family ($r=0.6041$, $p=0.039$) and *Prevotella* ($r=0.5809$, $p=0.039$) were positively correlated with liver TG concentration.

DISCUSSION

The purpose of this study was to comprehensively compare the adipose tissue (AT), vascular, gut, and systemic metabolic effects of an energy deficit attributed to voluntary exercise to that attributed to a reduction in total dietary calories in a rodent model of early life-onset obesity under Western HFD conditions. We tested the hypothesis that exercise by means of voluntary wheel running (i.e., Ex), when matched to an equal energy deficit caused by caloric reduction (i.e., WM), would lead to greater metabolic improvements and that Ex and WM would differentially affect gut microbial communities. Further, we determined whether changes in metabolic variables correlated with changes in gut microbial communities. The primary findings were that Ex was metabolically advantageous to WM in that it led to greater improvement in insulin resistance (IR), greater reduction in LDL cholesterol, increased spontaneous physical activity (SPA), increased fat oxidation, and increased BAT mitochondrial enzyme activity and content. However, counter to previous reports (8, 12), we did not detect evidence of Ex-mediated browning of WAT, nor did we see browning effects associated with WM. This lack of AT browning may be attributed to a suppressive effect of obesity on the browning process or that voluntary exercise is not a sufficient stimulus for

browning. These hypotheses should be addressed in future studies. Ex and WM were comparable in their abilities to improve body composition, reduce AT inflammation and whitening of BAT, improve blood lipid profile, and decrease vascular inflammation. Finally, both treatments modestly impacted the composition and abundance of gut microbial communities, with changes induced by Ex apparently more robust.

Body composition, energy balance, SPA, and fat oxidation

As expected, Ex significantly attenuated weight gain. We weight-matched this group to a separate group of sedentary, HFD-fed rats to better understand the energy deficit-independent effects of exercise. This was accomplished with a moderate reduction of 20-25% of *ad libitum* HFD. In light of mixed results in both animals and humans suggesting that exercise may (7) or may not (13) lead to a compensatory increase in energy intake, we measured energy intake of the Ex group fed *ad libitum* HFD. We found no Ex-mediated increase in energy intake, arguing against the hypothesis that exercise alone is ineffective in reducing body weight due to a compensatory increase in dietary energy, at least in this obesity-prone male rat model. Not only did Ex not increase food intake, but it increased SPA and total caloric expenditure (TEE). Interestingly, both energy deficits increased TEE (**Fig. 1E**). The increased TEE in the WM group was due to an increase in SPA only during the rodent inactive, rather than active cycle, whereas Ex increased SPA in both cycles. HFD has been shown to reduce SPA (40); the present data suggest that inducing an energy deficit by any means might lessen the SPA-reducing effect of HFD, and that Ex may be especially protective in this regard. Ex also increased fat oxidation, as indicated by lower RQ values in both cycles whereas WM did not affect fat oxidation; similar exercise-specific effects on RQ have been reported in humans (3).

Insulin sensitivity and fasting blood parameters

Exercise training is known to robustly enhance insulin sensitivity in both humans and animals, highlighting the efficacy of exercise as a therapeutic strategy. However, diet has also been shown to reduce IR when it is associated with weight loss (39). We found that, while both treatments improved glucose tolerance, only Ex improved insulin sensitivity. Neither treatment affected circulating NEFAs, which may suggest that the HFD-mediated IR was attributed more so to skeletal muscle IR than AT IR. Ectopic lipid deposition (e.g., increased liver TG), a common manifestation of AT IR (1), tended to decrease with both interventions. That this effect was only significant in the WM group was surprising since most evidence suggests that exercise reduces ectopic lipid deposition. The fact that differences did not exist in liver TG concentration or in circulating NEFAs between Ex and WM groups agrees with the hypothesis that the Ex-mediated improvement in systemic IR was due to skeletal muscle-specific improvements.

Adipose tissue and aorta inflammation

Confirmatory to previous findings (35), we found improved gene expression patterns across AT depots with both Ex and WM. Another study of young rats exposed to caloric deficit versus voluntary exercise reported similar improvements, but in that study, the exercise group had a greater attenuation in body weight, making it unclear whether the differences

between groups were due to the treatments *per se* or the differences in adiposity (8). In that study, exercise improved insulin-mediated vasodilation in the aorta. It could not be determined whether the protective effect of exercise on insulin-induced dilation was explained by the greater weight loss. While insulin-induced dilation was not assessed in the present study, we did find that Ex, not WM, improved systemic insulin sensitivity. Other studies (35) have compared similar energy deficits of exercise and diet (e.g., replacement of HFD with an LFD), but controlling for the exposure to dietary fatty acid composition is essential, since fatty acids profoundly impact AT inflammation. Moreover, the impact of replacing HFD with an *ad libitum* LFD has been shown to induce greater weight loss than exercise alone (35). Due to the higher energy density of the HFD, we were able to induce an energy deficit in the WM group without having to implement true caloric restriction (defined as 60-70% of *ad libitum* diet of normal energy density (15)). We found anti-inflammatory effects of both interventions in every AT depot investigated with the exception of PAT. In fact, despite its relationship with greater overall metabolic protection, Ex *increased* markers of oxidative stress in PAT. This may be attributable to an increase in mitochondrial-derived ROS, perhaps due to increased fat oxidation. That BAT citrate synthase activity and mitochondrial subunit content were greater in Ex compared to WM supports that contention. Unfortunately, those measures were not taken in PAT, which is a limitation of the study. Interestingly, ROS may actually stimulate insulin sensitivity and improve metabolic homeostasis (30). In PAT, the adiponectin to leptin ratio has been shown to be cardioprotective with greater adiponectin and lower leptin being associated with improved endothelial function (23). Here, the adiponectin to leptin ratio was improved with Ex only. Future work should determine if the mechanism by which Ex exerts vascular protection involves an improvement in immunometabolic changes in PAT. Importantly, both WM and Ex groups experienced reductions in both TNF- α and IL-6 in the aorta; thus, the apparent increase in PAT inflammatory gene expression with Ex did not appear to adversely affect the adjacent aorta. On the contrary, both interventions reduced inflammatory gene expression in aorta.

New data suggest that the BAT is metabolically more important than previously realized, and appears to be significantly affected by exercise (12), although there are conflicting reports on whether exercise increases (21) or decreases (41) its activity. We noted phenotypic differences in BAT independent of mass differences. As indicated, Ex significantly increased mitochondrial citrate synthase activity (by ~11%) in BAT, which may have contributed to the lower RQ values noted in the Ex group, as BAT is known to increase whole body fat oxidation (14). Both Ex and WM reduced pro-inflammatory markers in BAT, similar to the changes observed in the visceral WAT. However, BAT appeared more amenable to reductions in endoplasmic reticulum and oxidative stress markers, with both Ex and WM reducing markers of cell stress such as DDIT3, Hspa5, p22Phox, and p67Phox. This may be due to this depot's greater reliance on fatty acid oxidation and thus susceptibility to metabolic stress compared to other depots less dependent on mitochondrial fat oxidation (37). The fact that Ex increased expression of IL-13 (Th2 cytokine produced by M2 macrophages) and MRC-1 (mannose receptor expressed on alternatively activated macrophages) is of interest due to the recent controversy over whether exercise increases or decreases BAT activity (12). M2 macrophages, known to be activated with exercise (18),

have been shown to increase BAT activity (e.g., UCP-1 expression) and browning of WAT (29). The hypothesis that exercise may increase BAT function via M2 macrophage activation should be addressed in future studies. Counter to previous studies, BAT-related genes were not up-regulated in SQAT. This argues against the hypothesis that exercise induces WAT browning, at least in this model of HFD-induced obesity. That both leptin and adiponectin were reduced in BAT in Ex and WM groups suggests that these treatments lessened BAT-whitening associated with HFD.

Gut microbial changes

Gut microbiota have been linked to the development of obesity, with likely mechanisms pertaining to an alteration in energy harvest, modulation of gut peptides, and regulation of lipid metabolism (22). Despite lack of clear PCoA plot separation, we did find modest, yet statistically significant, effects of both Ex and WM on the composition and abundance of gut microbial communities. This is suggestive of subtle treatment effects as would be expected as opposed to differences that would be observed when comparing diseased vs. healthy individuals. Obesity has been associated with increased *Firmicutes:Bacteroidetes* ratio in mice and humans. Similar to our previous study (25), in this present study, Ex did not impact the species richness or the *Firmicutes:Bacteroidetes* ratio. One relative abundance difference was that S24-7 decreased in the Ex ($p<0.05$) but not the WM ($p=0.2$) group. This species has been shown to differ between diabetic and non-diabetic mice (19); thus, this Ex-related shift may be in line with the robust insulin-sensitizing effect of exercise. Opposite to previous observations reported by Kang et al., we observed an increase in the relative abundance of *Streptococcus* with exercise (17). We also observed that the relative abundance of the family *Streptococcaceae* was significantly increased with exercise. Compared to Sed, family *Rikenellaceae* decreased in the Ex group ($p<0.05$), but not in the WM group ($p=0.14$). This particular family positively correlated with liver TGs, which were improved in Ex and WM groups. The *Rikenellaceae* family has been associated with aging in mice and humans (20) and HFD in rodents (10). Overall, the relative abundance of *Prevotella* was positively associated with liver TG; this is potentially noteworthy since this organism has been shown to be positively associated with BMI and serum TG in adolescents (16). We also found that the relative abundance of *Peptostreptococcaceae* was positively associated with adiposity; similar findings were reported in a diet-induced obese mouse model (28).

A limitation of this study is that the gene expression differences observed across tissues were not all validated with protein measurements. At least, the current data suggest that there are similarities and differences between the effects of Ex and WM when the energy deficit is well controlled. Second, we did not directly assess BAT function by measuring cold-induced thermogenesis or utilizing PET scanning; thus, definitive conclusions about how the interventions may or may not affect BAT function cannot be drawn. Finally, this study was conducted in male juvenile rats, all of which were fed a HFD; as such, how the results translate to other rodent models or to humans is unknown. In summary, we compared cohorts of obesity-prone juvenile rats fed HFD who were either exercise-trained (Ex), or weight-matched to the energy deficit induced by voluntary exercise via limit-feeding (WM), in terms of a host of cardiometabolic disease risk parameters. We found that the two interventions were virtually equivocal in terms of improvements in body composition and in

reducing AT and aorta inflammation. However, only Ex had whole body insulin sensitizing benefits and enhanced systemic fat oxidation. Further, Ex did not result in a compensatory increase in energy intake, and actually *increased* SPA. While both energy deficit protocols improved BAT phenotype such that each reduced AT whitening (31), Ex also increased BAT mitochondrial enzyme activity and subunit density. Gut microbial composition and abundance appeared to be more affected by Ex than WM, and the changes observed correlated with improvements in some metabolic variables. However, the implications of these microbial changes, or the mechanisms by which they occur, require further investigation. Together, these results highlight the protective effects of dietary energy deficit and exercise training on a host of cardiometabolic parameters in juvenile obesity-prone rats during continued consumption of HFD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Body weight and adiposity

Body weight, adiposity, energy intake and expenditure, and insulin sensitivity. **A.** Body weight change over time; **B.** Final body weight; **C.** White adipose tissue (WAT) pad weights; **D.** Energy intake; **E.** Total energy expenditure; **F.** Spontaneous physical activity; **G.** Respiratory quotient; **H.** Resting energy expenditure; **I.** Matsuda index of insulin sensitivity; **J.** Homeostatic model assessment of insulin resistance (HOMA IR). SQAT = subcutaneous; EpiAT = visceral from epididymal region; RPAT = visceral from retroperitoneal region; OMAT = visceral from omental region; EE = energy expenditure. * $p < 0.05$ compared to Sed; # $p < 0.05$ compared to WM.

Figure 2. BAT phenotype differences

A. BAT Mass; **B.** BAT:WAT ratio; **C.** BAT citrate synthase activity; **D.** Respiratory Complexes; **E.** Correlation between MI and BAT CS Activity; **F.** BAT Activity related genes; **G.** SQAT genes associated with beige phenotype; **H.** BAT H&E Staining. * $p < 0.05$ compared to Sed (represented by line); # $p < 0.05$ compared to WM.

Figure 3. PCoA plots of weighted and unweighted UniFrac distances based on the UniFrac distance matrix generated from sequencing cecal 16S rRNA genes

A. Weighted PCoA; **B.** Unweighted PCoA. Each dot represents a sample from Sed (orange dots), Ex (red dots), and WM (blue dots) rats.

Table 1

Fasting Blood Markers and other Metabolic Measures.

Analyte	Sed	Ex	WM
Insulin (ng/mL)	7.3 ±1.0	4.1 ±1.2 ^{##}	7.8 ±1.1
Glucose (mg/dL)	179.0 ±6.6	163.4 ±6.8	192.2 ±12.3
Cholesterol (mg/dL)	78.5 ±5.4	60.3 ±4.8 [*]	61.8 ±2.8 [*]
Triglycerides (mg/dL)	165 ±22.2	133 ±27.0	166 ±12.3
HDL (mg/dL)	22.0 ±1.1	19.7 ±1.1	21.4 ±0.9
LDL (mg/dL)	5.7 ±0.4	4.1 ± 0.3 ^{##}	6.5 ±0.3
NEFA (mmol/L)	0.6 ±0.1	0.6 ±0.1	0.7 ±0.1
%HbA1c	4.33±0.02	4.27±0.04	4.2±0.03 [*]
Glucose AUC	30551±4090	25082±5769 [*]	23518±3063 [*]
Insulin AUC	198±80	111±87 [*]	177±33
Liver Mass (g)	15.54±0.37	12.77±0.64 [*]	15±0.39
Liver TG (mg/g wet weight)	46.10±2.48	37.82±7.52	27.12±1.87 [*]

All measures were taken at sacrifice after ~12-h fast. HDL = high density lipoprotein cholesterol; LDL = low density lipoprotein cholesterol; NEFA = nonesterified fatty acids; %HbA1c = glycosylated hemoglobin; glucose, insulin AUC = glucose, insulin area under the curve during glucose tolerance test which was performed at 12 weeks of age.

^{*} Denotes difference from Sed;

[#] denotes difference from WM (p<0.05).