



Exercise training prevents high-fat diet-induced adipose tissue remodeling by promoting capillary density and macrophage polarization

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ABSTRACT

Aims: Increasing adipose tissue capillarization is beneficial to metabolic health. The present study examined the effects of continuous training (CT) and aerobic-interval training (AIT) coinciding with a high-fat diet (HFD) on capillary density, macrophage polarization in mesenteric (mAT) and subcutaneous (sAT) adipose tissue.

Main methods: 48 male Wistar rats were divided into HFD and normal diet (ND) groups. After 10 weeks, each group was divided into sedentary, CT, and AIT. The animals in training groups performed 10-week matched distances of CT and AIT on a motorized treadmill (5 times/week).

Key findings: The results showed that HFD significantly reduced the capillary density of adipose tissue (sAT: 54% vs. mAT:49%) and increased systemic insulin resistance, mean adipocyte size, crown-like structure (CLs), and M1-macrophages with no change in the total number of adipocytes in either tissue. Exercise training reversed the HFD induced adipose tissue dysfunction. Compared to CT, AIT was more effective on increasing the capillary density of sAT (170 vs. 87%) and mAT (140 vs. 100%). Likewise, AIT increased the capillary density of both tissues even in comparison to the ND sedentary group (~25%). Compared with CT as well, AIT more significantly increased the number of M2 macrophages (181 vs. 122%) and decreased CLs (60 vs. 38%) in mAT.

Significance: The findings suggest that hypertrophy is a major contributor to adipose tissue expansion in obesity. Furthermore, exercise training largely demonstrated beneficial effects on adipose tissue remodeling, where AIT is more effective than CT in reducing HFD-induced adipose tissue dysfunction.

1. Introduction

The chronic excess of caloric intake leads to obesity and contributes to the development of cardiovascular diseases and diabetes. Adipose tissue (AT) neutralizes the risk factors associated with the excessive energy intake via adipocytes hypertrophy or hyperplasia [1,2]. However, the normal AT expansion depends on the coupling of adipogenesis and angiogenesis [3,4]. Moreover, the disparity between adipogenesis and angiogenesis may result in AT hypoxia [4]. Hypoxia entails the death of adipocytes and is further linked to inflammatory macrophage infiltration and polarization from M2 to M1 [4,5]. It is also suggested that inflammatory macrophages (M1) are closely related to insulin resistance [6].

In addition, it has been well established that the pharmacological increase in AT angiogenesis is accompanied by a normal expansion of AT, lower inflammation and metabolic dysregulation [3–6]. Therefore, the increase in AT angiogenesis can be considered as a therapeutic option for the treatment of obesity-related complications such as insulin

resistance.

Aerobic exercise training as a therapeutic intervention ameliorates adipose tissue inflammation, metabolic disturbances [7–9], and systemic insulin resistance [7,10] in obese subjects. Previous studies have revealed that moderate-intensity continuous training enhances capillary density in subcutaneous (sAT) and epididymal adipose tissue (eAT) of healthy rodents and humans [11,12]. However, it has no effects on sAT capillary density in insulin resistance subjects [11]. Therefore, it seems that higher intensity exercises are required to induce AT angiogenesis in obese subjects.

It is clearly documented that maximal and supra-maximal interval training with short intervals (10–60 s) have metabolic benefits, equal or higher than continuous training (CT) [13,14]. Nonetheless, these types of exercise might not be well tolerated by clinical populations. Several publications [7,14] have recommended aerobic-interval training (AIT) with longer periods (3–4 min/bout) to improve the metabolism of skeletal muscle and AT in clinical subjects. Although the mechanisms of such changes in the AT are unclear, most studies have reported that

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moderate-intensity continuous training reduces adipocytes hypertrophy and switches M1 toward M2 (anti-inflammatory) macrophages in the eAT of rodents [1,9,10,15]. Unfortunately, most studies have considered eAT to represent visceral adipose tissue (VAT) [1,15,16], whereas eAT does not exist in humans. Similar to human VAT, the venous return of mAT in rodents drains into the portal vein and returns fatty acids and inflammatory cytokines to the liver [17,18]. This may explain why the accumulation of lipid in the mAT of rodents and human is positively associated with insulin resistance of the liver, and, potentially, of skeletal muscle [17,19]. Thus, in response to long-term excess energy intake and exercise training, evaluating the changes in the mAT of rodents may provide a better insight into the VAT of human.

To examine the aforementioned theory, the interrelationships among exercise training, angiogenesis, inflammation, and obesity in response to long-term high-fat diet (HFD), AIT and CT in male Wistar rats were evaluated. The hypothesis is that exercise training through angiogenesis induction prevents abnormal adipose tissue expansion and inflammation during obesity.

2. Materials and methods

2.1. Animal care

Animal care and ethical principles were based on the Guide for the Care and Use of Laboratory Animals [20] approved by the Ethics Committee of University of Mazandaran. To this end, 48 male Wistar rats (4–6 weeks old) were purchased from Tehran Razi Institute, and housed in cages (4 rats per cage) at room temperatures of 21–23 °C (12-hour light, 12-hour dark). Following a week of adaptation to the environment, the rats were divided into HFD and Normal diet (ND) groups (phase 1). During phase 1 (10 weeks), the rats had free access to HFD (62.1% kcal from fat) and ND (10.4% from fat) [21]. The fat used in HFD regime was obtained from the rump fat in sheep. Briefly, after melting the rump fat, it was mixed with carbohydrates and protein in a mixing machine for 30 min, and was then converted to pellet using an automatic machine. The rump was analyzed by Gas Chromatography Mass Spectrometry (GC/MS: Agilent technologies, USA). Kjeldahl and Fehling's methods were used for the quantitative determination of protein and carbohydrates in the diets. Moreover, the total diet fat was measured by Soxhlet extraction method. The diets' composition is provided in Table 1.

After 10 weeks of HFD or ND, each group was divided into sedentary (S), CT and AIT (phase 2; 10 weeks, 5 times/week) based on body weight. The HFD group continued their free access to HFD (fresh HFD) during phase 2. The body weight and food intakes were recorded every week.

2.2. Training protocols

In phase 2, after a week of adaptation to treadmill running (3 times/week, 10-min/session), AIT groups performed 3-min bouts at 40 m/min, interspersed by 3-min active recovery at a running velocity of

20 m/min on a motorized treadmill with 15% incline, repeated six times per session (total time: 36 min). The duration, velocity, and incline were gradually increased over a period of 6 weeks to 36 min/session, 40 m/min, and 15%, respectively. The CT groups performed steady state running at the same speed as the active recovery's speed in the AIT group, where the running distances of CT groups were matched based on running distances in AIT sessions over time. Each training session further consisted of 10-min warm-up and cool-down (the speed; 10-m/min at 0% incline). The training protocols were slightly modified relative to previous studies [22,23], where the speed of 40 m/min at a 15% incline elicited approximately $\geq 85\%$ VO_{2Peak} [22,23]. The exercise training program was done in the active cycle of rats (dark cycle). In order to reduce the stress of the cages, the sedentary rats were kept out of the cages on a stationary treadmill for the same period of exercise as the training groups.

2.3. Adiposity index and plasma variable measurement

After spending a 20-week period of treatment, the rats in the fasting state (8 h) were anesthetized 48 h right after the last exercise session. Blood samples were driven from the heart and centrifuged and separated immediately. For future analysis, Plasma samples were kept frozen at -20°C . Insulin was measured via ELISA kit (Merckodia, Uppsala, Sweden). Glucose, lipid profile, and blood immune cells were measured through the use of Auto Analyzer (Hitachi, Japan and BC-5800, China respectively).

Furthermore, fat pads (Inguinal, Mesenteric, Perirenal, Retroperitoneal, and Epididymal) and liver were removed and weighed. Adiposity index was further calculated based on fat pads weight (Inguinal, Retroperitoneal, and Epididymal) and body weight [24].

2.4. Tissue sampling, sectioning, and histological analysis

For microscopical studies, the entire mAT and sAT (inguinal) were removed and weighted, after which process, 1 g of the adipose tissues was fixed in isotonic neutral buffered 10% formalin solution. Prior to tissue processing, the total volume of samples was calculated by Scherle methods, in which, briefly, a jar filled with isotonic saline is placed on a precision scale and weighed. Next, each sample is suspended by a thin thread and is fully immersed, but does not touch the bottom or the side of the jar. After that, the volume is estimated by $V = (W_2 - W_1) / \sigma$, in which “ W_2 ” and “ W_1 ” correspond to the weight of the saline filled jar, before and after adipose tissue immersion, respectively, and “ σ ” is the specific gravity of isotonic saline (1.0048 g/cm^3). The weight in grams is equal to the volume in cm^3 [25]. Finally, the specific gravity of mAT and sAT was obtained through dividing 1 by the volume of the sample (g/cm^3); the total volume (V_{ref}) of mAT and sAT in each animal was further calculated.

Fixed tissues were dehydrated in graded alcohol series, cleared in xylene, impregnated in paraffin wax and embedded in paraffin block according to an isotropic uniform random (IUR) [26]. Using a rotary microtome, each block was cut into four 20 μm thick serial sections, and

Table 1
Nutritional composition of the diets.

	Normal diet		High fat diet	
	g	kcal	g	kcal
Kcal/g	–	3.71	–	5.05
Carbohydrates	60%	64.4%	35.7%	28.2%
Protein	23.1%	24.8%	20%	16%
Total fat	4.3%	10.4%	31.4%	62.1%
Fat ingredients	Saturated fatty acids 37.3%		Unsaturated fatty acids	
			62.69%	
		Monounsaturated 71.62%	Trans monounsaturated 23.12%	Polyunsaturated 5.21%

then four 5 μm thin serial sections, a process continued to the end of tissue in each block. Sections were collected on slides and stained with hematoxylin-eosin (H&E) and Mason's trichrome. Systematic random sampling protocol was performed for sampling, where the first section was selected randomly. Next, five thin sections from each block were histologically analyzed to evaluate adipose tissue architecture and crown-like structures after staining with H&E as described previously [27]. Finally, 20 to 25 thick sections and five thin sections were further selected from each block for stereological and immunohistochemical analysis, respectively.

2.5. Stereological analysis

In this study, we aimed at developing an efficient and practical design-based combination of stereological tools for the estimation of various parameters of mAT and sAT structure in rats. Stereological investigations provide information on volume and, via certain extended techniques (3D sample), the number of structural components. It is a gold standard for morphometric analysis of adipose tissue. Stereological studies were carried out strictly under blind conditions with optical fractionator for estimating the volume fraction (V_v), the numerical density (N_v) and the mean cell volume of adipocytes (VA) in mAT and sAT by use of stereo-investigator system (Version 9, MBF Bioscience, MicroBrightField, Inc., Germany). This system consists of a standard microscope, a motorized stage, digital camera and a software program which commands automatic XY displacements of the microscope stage, allowing the systematic randomized sampling of tissue microscopical fields. The program further generates point grids, dissectors, and nucleators, superimposed on the tissue samples visualized on a monitor.

2.6. Estimation of the volume fraction (V_v) and absolute volume of adipocytes, and blood vessels

A systematic randomized sampling of microscopical fields was made at $40\times$ magnification in each section, and a 100-point grid was superimposed on each microscopical field. The volume fraction (V_v) of adipocytes, blood vessels, was calculated by [28]: $V_v(\text{structure}/\text{ref}) = P_{(\text{structure})} / P_{(\text{total})}$ where " $P_{(\text{structure})}$ " is the number of points hitting the profiles of adipocytes, blood vessels, and " $P_{(\text{total})}$ " is the number of points hitting the section. Finally, the absolute volume of each structure was obtained by multiplying its volume fraction by the total volume (V_{ref}).

2.7. Estimation of the numerical density (N_v) and the total number of adipocytes

The relative number of adipocytes was estimated by the optical disector principle [29]. Disectors were generated as successive focal planes inside a thick section of adipose tissue. A high numerical aperture oil immersion lens was used. By means of the stereological software, an unbiased counting frame was superimposed on the sections, and microscopical fields were selected by systematic randomized sampling. The number of adipocytes was counted by considering their nuclei as the counting unit (each corresponding to only one adipocyte). Only adipocytes within the unbiased counting disector frame and satisfying the Stereo rule were counted. Finally, the number of adipocytes was estimated using optical disector method and the following formula [30]: $N_{v(\text{cell}/\text{ref})} = \Sigma Q^- / \Sigma A \times h$ where " ΣQ^- " is the number of cells coming into focus in the disector height, " ΣA " is the total area of the unbiased counting frame in all microscopic fields and " h " is the height of disector. The total number was further estimated by multiplying the numerical density by V_{ref} .

2.8. Estimation of the mean cell size of adipocytes (VA)

The nucleator method was used for obtaining the mean cell size of

adipocytes as previously described [31]. At $20\times$ magnification, a reference point is marked at the geometrical center of the adipocyte, through which the stereological program produces two perpendicular axes, intersecting the cellular membrane of the adipocyte at four points. These four intersections are marked, and the program estimates the size of the structure (expressed in μm^3) based on the formula: $VA = 4/3 \pi l_o^3$, considering " l_o " as the mean length of the segments between the reference point and membrane intersection point. Finally, the mean cellular size of the adipocytes (VA) is estimated as the total size of the adipocytes (ΣVA) sampled for the measurement and divided by the total number of sampled adipocytes (n): $VA (\mu\text{m}^3) = \Sigma VA / n$.

2.9. Immunohistochemical analysis for the evaluation of infiltrated macrophages

These selected 5 μm tissue sections were collected on a silanized slide (Dako, S3003), deparaffinized in xylene and rehydrated in a series of graded ethanol solution and immunostained as described previously [32]. Briefly, heat mediated antigen retrieval was performed in 10 mM citrate buffer (10 mM tri-sodium citrate, 10 mM citric acid, pH 6.0) with slides microwaved at 800 W for 4 min, and 160 W for 10 min, and subsequently cooled at room temperature for 20 min. Next, the tissue sections were delineated using a Dako pen (Dako, Glostrup, Denmark), rinsed in PBS and incubated with 3% hydrogen peroxide in methanol for 1 min so as to eliminate endogenous peroxidase activity. After that, slides were washed in PBS before being treated with 0.1% Triton X-100 in citrate buffer, and then washed again and treated with 50% goat serum in PBS for 30 min. After rinsing in PBS, the tissue sections were incubated with anti-CD86 antibody to detect M1 macrophages (ab53004, diluted 1:100 in antibody diluent with 5% goat serum) or anti-CD206 antibody for the detection of M2 macrophages (ab64693, diluted 1:100 in antibody diluent with 5% goat serum). Afterwards, they were each washed three times for 5 min with PBS, followed by incubation with secondary antibody biotinylated goat anti-rabbit IgG (Vector BA-1000, diluted 1:200) diluted in 5% goat serum applied and incubated for 30 min at room temperature. The secondary antibody was washed off in PBS and avidin-biotin complex reagent prepared according to the manufacturer's directions and applied to each section for 30 min followed by rinsing in PBS. The antibody was visualized with 3,3-diaminobenzidine tetrachloride (DAB) at room temperature for 2 min, washed in distilled water and lightly counterstained with Harris hematoxylin for 2 min. Slides were subsequently dehydrated and cleared in xylene prior to being mounted in Vectashield mounting medium (Vector Laboratories, Inc.) with a coverslip for microscopical analysis. Five fields from each section were photographed using an Olympus BX-60 microscope with an Olympus DP 12 digital camera (Olympus Corporation, Japan); immunostained macrophages were then counted in a blinded manner. A minimum of 400 adipocytes were examined for each sample, and finally, the number of macrophages was normalized for 100 adipocytes.

2.10. Immunofluorescence analysis for the evaluation of capillary density

The selected 5 μm tissue sections on the silanized slide (Dako, S3003) were deparaffinized and rehydrated as portrayed above, and immunostained as described previously [33,34]. Briefly, tissue samples were digested with 20 mg/mL proteinase K for 5 min, followed by incubation in methanol for 30 min. The permeabilized tissues were stained with an anti-CD31 antibody (ab28364; 1:300 dilution) at 4 $^{\circ}\text{C}$ overnight. After rinsing three times in PBS, the sections were incubated with Alexa 555-conjugated anti-rat secondary antibody (1:500 dilution) at room temperature for 2 h. After washing in PBS, slides were dehydrated, cleared in xylene, and mounted in Vectashield mounting medium (Vector Laboratories, Inc.) with a coverslip, and then examined under a confocal microscope with a Olympus DP 12 digital camera (Olympus Corporation, Japan) at $400\times$ magnification for capillary

density analysis; further photomicrographs were analyzed by ImageJ software (version 1.49) to quantify immunofluorescence reaction for CD31 expression according to previously described methods [35]. Capillaries were identified by positive staining for CD31 and counter stained with Harris hematoxylin. For analysis, digital photomicrographs of the entire adipose tissue sections were used in five different regions, and to assess the regional heterogeneity in tissue samples; regions were outlined using ImageJ software. The number of positive pixels was normalized to the total number of (positive and negative) pixels to account for variations in the size of the sampled region. In order to detect immunostaining as positive and background staining as negative pixels, color and intensity thresholds were established. Finally, after establishing the conditions, all slides were analyzed using the same parameters. The resulting color markup of the analysis was confirmed for each slide. The capillary density was determined by counting the total number of CD-31 positive capillaries, with diameter < 20 μm , in an AT section, and expressed as the number of capillaries per square millimeter.

2.11. Statistical analysis

Data values are expressed as a mean \pm standard error of means (SEM). All data were analyzed with 2 ways ANOVA (Diet \times exercise). To establish a comparison within the groups (HFD or ND), one way ANOVA and Bonferroni's post hoc test were applied. Pearson's correlation was also utilized to determine the relationship between variables. Moreover, the paired student test was used to compare mAT and sAT morphology. The significant differences were considered at $P < 0.05$.

3. Results

3.1. The effects of HFD and exercise training on body weight and adiposity index

The results showed that the body weight was significantly higher and gradually increased during HFD feeding, compared to that of ND (Fig. 2A). Furthermore, the fat mass and adiposity index of HFD fed rats were significantly greater than those of ND. In comparison with HFD fed sedentary rats (HS), CT and AIT significantly attenuated body weight (30 and 40%, respectively), fat mass and adiposity index increases induced by the HFD (Table 2, $P < 0.05$).

3.2. The effect of HFD and exercise training on the adipocytes size and number of sAT and mAT

Sections of sAT and mAT were stained with hematoxylin and eosin to demonstrate the general histology of adipose tissue (Fig. 1). The data illustrated that after HFD or ND feeding, sAT had a significantly higher total adipocytes number than mAT (Fig. 2B, $P = 0.001$), although there was no significant difference between the two diets. Mean adipocyte size of mAT was greater than sAT in HFD fed rats (Fig. 2C, $P = 0.001$). Both exercise protocols reduced the total number of sAT adipocytes after HFD or ND feeding (Fig. 2D, $P < 0.001$). However, AIT and CT only decreased the total number of mAT adipocytes in the ND regimen (Fig. 2F, $P < 0.001$).

HFD increased the mean adipocyte size of sAT and mAT, which were diminished by AIT and CT (Fig. 2E, G, $P < 0.001$). AIT had a greater impact on sAT mean adipocyte size reduction compared with CT (Fig. 2E, $P < 0.001$). Overall, exercise training prevented the effects of high-fat diet on adipose tissue cell structure changes, which was more effective in sAT than mAT.

3.3. The effect of HFD and exercise training on sAT and mAT capillary density

Fig. 3 shows the representative image of the capillary density

analysis by Immunofluorescence method. Using CD31 (endothelial cell surface marker) antibody, as observed, mAT had a greater baseline capillary density than sAT ($P = 0.034$), and, after HFD feeding, it was significantly diminished by 54% ($P < 0.001$) and 49% ($P = 0.001$), respectively (Fig. 4A, $P < 0.05$).

In comparison to CT, AIT more significantly increased the capillary density of sAT (170% vs. 87%; $P < 0.001$) and mAT (140 vs. 100%; $P = 0.004$) compared to that of HS (Fig. 4B and C). In ND group, on the other hand, AIT increased only the sAT and mAT capillary density (Fig. 4B, C, $P < 0.005$). Altogether, the exercise training effects were significantly more pronounced in AIT than in CT even in the presence of high-fat diet or normal diet.

3.4. The effect of HFD and exercise training on sAT and mAT macrophage polarization, and crown-like structure

So as to specify macrophage polarization (M1 and M2), the issue sections were analyzed by immunohistochemistry method (Supplementary Figs. S1 and S2). The results revealed that mAT had significantly higher M1 macrophages than those of sAT after administering HFD (Fig. 5A, $P < 0.016$). Interestingly, there was no significant difference regarding the number of M1 macrophages between the two tissues in the ND regimen, and M2 macrophages in HFD or ND regimens (Fig. 5A and D, $P > 0.05$).

HFD-induced CLs accumulation and macrophage polarization from M2 to M1 in sAT and mAT were reversed by exercise training (Fig. 5A–I, $P < 0.001$), whereas AIT had a significantly greater influence on reducing CLs formation, and increasing the number of M2 macrophages even in the presence of HFD (Fig. 5E, F, H and I, $P < 0.05$).

The number of M1 macrophages in sAT and mAT of ND groups was significantly decreased following exercise training (Fig. 5B and C, $P < 0.002$). Moreover, in sAT and mAT, only AIT was able to increase the number of M2 macrophages following ND feeding (Fig. 5E and F, $P < 0.002$).

Also, the ratio of M1 to M2 macrophages in sAT and mAT was significantly increased by HFD ($P < 0.001$, $P = 0.001$, respectively), which was markedly reversed in the HFD-fed rats subjected to AIT or CT ($P < 0.001$). In addition, the results showed that there was no significant difference in the ratio of M1/M2 macrophages between sAT and mAT in the HFD-fed rats ($P = 0.07$). Overall, AIT effects on macrophage polarization were more pronounced in both tissues compared with CT effects.

3.5. The effect of HFD and exercise training on lipid profiles and systemic insulin resistance

Although HFD increased plasma triglyceride (TG), it had no effects on HDL, LDL, and TC (Table 2). Likewise, both AIT and CT decreased the TG concentration in the ND group, contrary to the HFD group. Despite HFD feeding, AIT and CT significantly mitigated TC plasma concentration ($P = 0.006$). Furthermore, HFD had no effects on circulating immune cells ($P > 0.05$, Table 2).

After 20 weeks, HFD significantly increased systemic plasma glucose ($P = 0.002$) and HOMA-IR ($P = 0.039$) compared to ND. While systemic circulating insulin tended to increase, HFD had no significant effects on systemic insulin concentration (Table 2, $P = 0.133$). CT and AIT reduced the plasma glucose, insulin, and HOMA-IR induced by HFD ($P < 0.05$). In the normal diet regimen, only AIT decreased plasma insulin and HOMA-IR compared with the sedentary group (Table 2, $P < 0.007$).

3.6. The correlation between plasma variable and adipocyte morphology

Capillary density of AT was positively correlated with M2 macrophages, yet negatively associated with M1 macrophages and glucose homeostasis (Table 3). Furthermore, the number and mean adipocyte

Table 2
Animal characteristics and plasma variables.

	NS	NCT	NIT	HS	HCT	HIT
Body weight before training (g)		369.34 ± 4.71			403.34 ± 5.10	
Final weight (g)	418.57 ± 12.3	384.25 ± 10.4	380.75 ± 9.3	505.37 ± 11.4*	414.37 ± 10.4	421.14 ± 8.0†
BMI (Kg/m ²)	0.71 ± 0.01	0.65 ± 0.01‡	0.67 ± 0.01	0.80 ± 0.01*	0.68 ± 0.01†	0.71 ± 0.009†
Adiposity index (%)	6.28 ± 0.42	2.85 ± 0.24‡	2.50 ± 0.14‡	9.04 ± 0.69*	5.28 ± 0.53†	4.84 ± 0.36†
Food intake during training (g/an animal/day)	17.28 ± 0.19	16.21 ± 0.16	15.78 ± 0.15	17.15 ± 0.11	13.18 ± 0.18†	14.65 ± 0.15
Liver weight (g)	12.52 ± 0.40	10.83 ± 0.43‡	10.49 ± 0.25‡	15.72 ± 0.30*	13.29 ± 0.89†	13.02 ± 0.23†
Fat pads' weight (g)						
Inguinal	11.37 ± 0.99	4.42 ± 0.40‡	3.55 ± 0.20‡	16.68 ± 1.53*	7.22 ± 0.68†	6.88 ± 0.55†
Mesenteric	5.74 ± 0.68	2.01 ± 0.43‡	1.72 ± 0.08‡	9.20 ± 1.23*	3.33 ± 0.35†	3.44 ± 0.4†
Epididymal	8.59 ± 1.06	4.31 ± 0.37‡	4.12 ± 0.24‡	13.71 ± 1.55*	7.93 ± 0.73†	7.01 ± 0.45†
Retroperitoneal	6.56 ± 0.57	2.24 ± 0.29‡	1.78 ± 0.35‡	15.74 ± 1.68*	6.80 ± 1.00†	6.49 ± 0.68†
Prerenal	2.86 ± 0.37	1.01 ± 0.17‡	0.75 ± 0.04‡	4.57 ± 0.42*	2.15 ± 0.19†	1.84 ± 0.13†
Plasma profile						
TG (mgdL ⁻¹)	106.71 ± 12.2	54.50 ± 6.1‡	54.50 ± 2.2‡	154.25 ± 16.0*	138.62 ± 12.7	131.00 ± 17.5
HDL (mgdL ⁻¹)	32.71 ± 4.3	31.75 ± 3.2	32.37 ± 3.4	32.75 ± 4.5	25.87 ± 1.8	27.14 ± 2.3
LDL (mgdL ⁻¹)	51.98 ± 6.7	43.1 ± 6.1	41.06 ± 4.7	46.58 ± 8.7	32.8 ± 3.9	32.5 ± 4.8
TC (mgdL ⁻¹)	92.71 ± 5.8	78.12 ± 3.2	81.37 ± 3.8	97.75 ± 7.8	74.50 ± 1.06†	76.85 ± 2.9†
Glucose (mgdL ⁻¹)	107.38 ± 2.5	97.65 ± 4.2	97.65 ± 1.7	120.22 ± 1.5*	110.55 ± 1.5†	111.25 ± 1.7†
Insulin (mU/mL)	5.79 ± 1.04	4.18 ± 0.16	2.92 ± 0.15‡	8.04 ± 0.93	3.86 ± 0.33†	3.79 ± 0.13†
HOMA-IR	1.49 ± 0.25	0.99 ± 0.05	0.69 ± 0.04‡	2.35 ± 0.27*	1.04 ± 0.09†	1.03 ± 0.03†
WBC (10 ⁹ /L)	5.49 ± 0.35	5.82 ± 0.48	4.86 ± 0.35	6.40 ± 0.41	5.20 ± 0.35	5.64 ± 0.58
Neutrophil (10 ⁹ /L)	1.60 ± 0.24	1.32 ± 0.12	1.31 ± 0.14	1.59 ± 0.09	0.92 ± 0.18	1.51 ± 0.44
Lym (10 ⁹ /L)	3.72 ± 0.39	4.35 ± 0.42	3.35 ± 0.31	4.61 ± 0.32	3.36 ± 0.44	2.97 ± 0.70
Platelet (10 ⁹ /L)	804 ± 18	623 ± 19	687 ± 94	883 ± 45	621 ± 30†	758 ± 31

Data are represented as mean ± SEM.: *n* = 8 animal/group. Normal diet plus sedentary, NS; Normal diet plus continuous training, NCT; Normal diet plus interval training, NIT; High-fat diet plus sedentary, HS; High-fat diet plus continuous training, HCT; High-fat diet plus interval training, HIT; *vs. NS; †vs. HS; ‡vs. NS, (*P* < 0.05).

size were positively correlated with M1 macrophages, but negatively related to M2 macrophages (Table 3, *P* < 0.05).

The capillary density of sAT was inversely correlated with sAT adipocyte size (*r* = −0.851, *P* < 0.001) and number (*r* = −0.526, *P* = 0.003). Similarly, the capillary density of mAT was correlated with mAT adipocyte size (*r* = −0.911, *P* < 0.001) and number (*r* = −0.372, *P* = 0.043).

Plasma glucose had a significantly positive and strong correlation with M1 macrophages in sAT and mAT (*r* = −0.717, *P* = 0.001; *r* = −0.735, *P* < 0.001). However, plasma glucose merely had a significant inverse correlation with mesenteric M2 macrophages (*r* = −0.453). Furthermore, HOMA-IR had a significantly positive relationship with M1 macrophages (*r* = 0.584, *P* = 0.001; *r* = 0.515, *P* = 0.004), but a negative correlation with M2 macrophages in sAT and mAT (*r* = −0.661, *P* < 0.001; *r* = −0.751, *P* < 0.001).

Plasma insulin had a significant inverse correlation with M2 macrophages (*r* = −0.638, *P* < 0.001; *r* = −0.712, *P* < 0.001) and a positive correlation with M1 macrophages in sAT and mAT, respectively (*r* = 0.476, *P* = 0.008; *r* = 0.410, *P* = 0.024). Glucose had a strong positive correlation with sAT and mAT CLs (*r* = 0.625, *P* < 0.001; *r* = 0.628, *P* < 0.001), and a modest positive correlation with HOMA-IR (*r* = 0.523, *P* = 0.003; *r* = 0.498, *P* = 0.005). Furthermore, glucose, insulin, and HOMA-IR had a significantly positive association with sAT and mAT mass, (*P* < 0.001). Altogether, the capillary density of adipose tissue was closely related to adipose tissue inflammation and glucose homeostasis.

4. Discussion

This study is the first to evaluate the effects of different exercise training protocols on mAT and sAT remodeling in diet-induced obese rats. We showed that AIT and CT, even in the presence of HFD, reversed HFD-induced mAT and sAT dysfunction in part by reducing adipocytes size, increasing capillary density and switching macrophages from M1 to M2, ultimately lowering insulin resistance.

Expansion of adipose tissue during obesity is caused by the increase in the size and number of adipocytes [9]. The present study shows that

adipocytes hypertrophy significantly conduces to AT expansion during obesity, which is more pronounced in mAT than sAT. As previously suggested, the number of eAT adipocytes increases in response to a high-fat diet [2]. However, our findings showed no evidence for the increase in the number of adipocytes during obesity. It should be further noted in Fig. 2G that the number of mAT adipocytes was slightly reduced by HFD, a discrepancy possibly explained by the differences in depot-specific biology and physiology [2,17,18]. Therefore, this result may suggest that mAT and eAT have different responses during obesity. It was further observed that HFD-induced increase in the mean adipocyte size and CLs accumulation in the mAT were higher than sAT. It has been reported that CLs is positively correlated with hypertrophied and dead adipocytes [6]. As previously suggested [2], these findings may indicate that mAT adipocytes are more likely susceptible to apoptosis. Thus, it appears that the total number of adipocytes remains intact, yet dead adipocytes are replaced by new cells during obesity. Accordingly, adipocytes may respond to nutrient excess through hypertrophy rather than hyperplasia.

On the other hand, exercise training reduces body weight and AT mass by reducing the mean adipocyte size and number in obese subjects [9,10,12]. In this regard, the present study revealed that AIT and CT prevented body weight gain and fat mass by decreasing the mean adipocytes size and number. Despite the presence of HFD, exercise training preserved body weight at the basal level. Additionally, AIT had more significantly reduced the mean adipocyte size compared to the CT (Fig. 2F and I). It seems that the effect of exercise training on adipocytes size may be mediated by an increase in lipid oxidation and a decrease in lipogenesis [10,12]. Another mechanism is likely related to AT capillary density. In agreement with previous reports [4,11], the results of the present study indicated a strong inverse correlation between the mean adipocyte size and capillary density of AT. Moreover, the basal capillary density of mAT was higher than sAT (Fig. 4A). However, in this study, during energy surplus, the capillary density of mAT and sAT was reduced approximately 54 vs. 49%, corroborating the AT hypoxia concept during obesity [16,36,37].

The present findings showed that AIT and CT coinciding with HFD augmented the capillary density of sAT and mAT in diet-induced obese

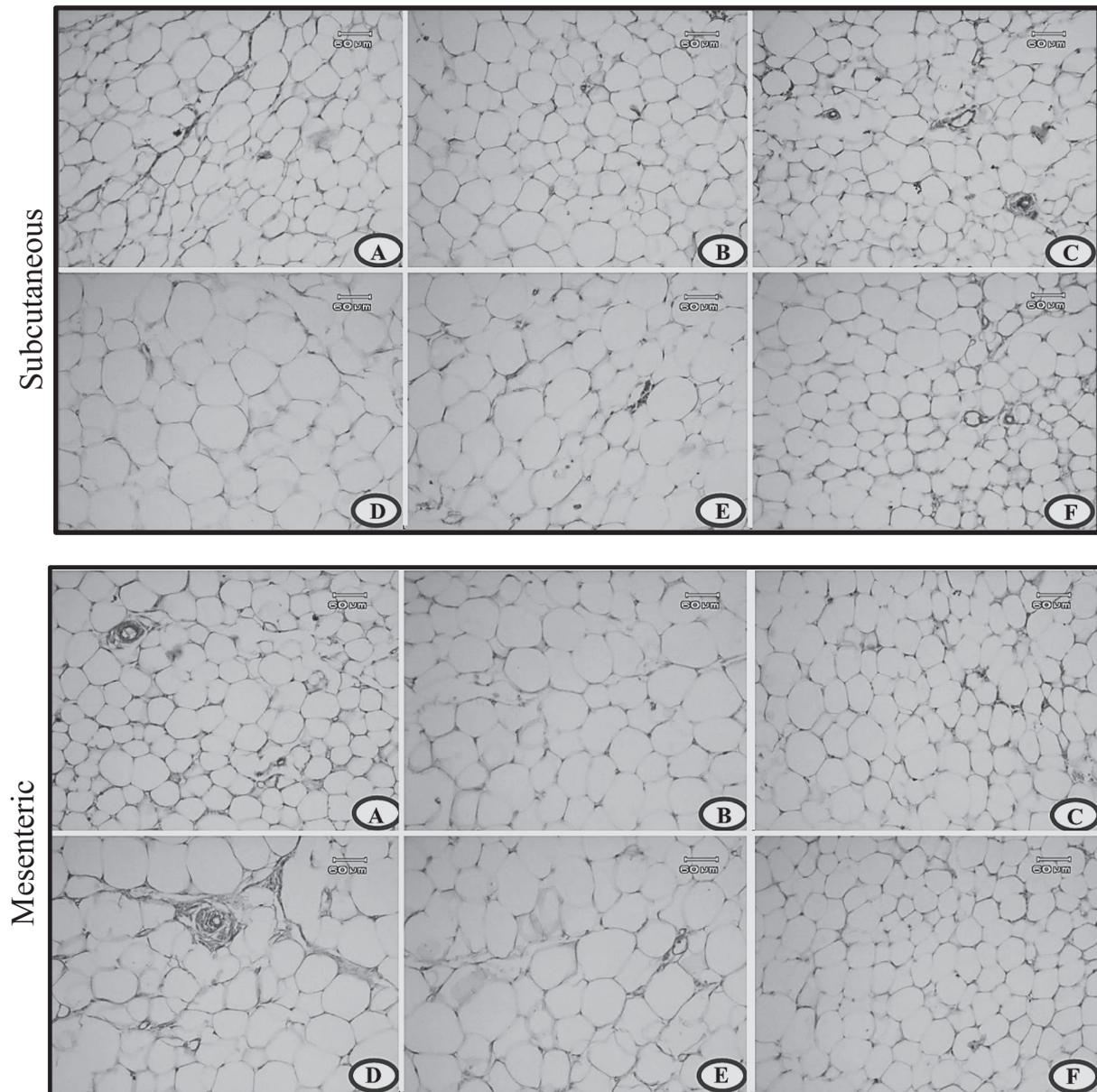


Fig. 1. Sections of mesenteric adipose tissue were stained with hematoxylin and eosin to demonstrate the general histology of adipose tissues (scale bar, 60 μ m). A; normal diet sedentary, B; normal diet plus continuous training, C; normal diet plus aerobic interval training, D; high-fat diet sedentary, E; high-fat diet plus continuous training, and F; high-fat diet plus aerobic interval training.

rats. Furthermore, compared to CT, AIT led to a greater increase in the capillary density by 170 vs. 87% and 140 vs. 100% in sAT and mAT, respectively. In addition, compared to mAT, sAT had a higher angiogenic capacity which can be influenced by training intensity, a finding consistent with the higher angiogenic capacity of sAT compared to VAT in humans [36]. It can therefore be speculated that rodent mAT, rather than eAT, is likely more similar to human VAT. Although this speculation requires further investigations, it should be considered in the design of future studies. In addition, the results showed that AIT significantly enhanced the capillary density in both sAT and mAT in ND-fed rats. Previous studies have shown that moderate-intensity continuous training increases the capillary density of sAT in humans and eAT in healthy rodents [11,12], but not in the sAT of insulin resistance subjects [11]. The results suggest that the extent of adipose tissue remodeling response to exercise training is influenced by exercise intensity. To confirm this speculation as the novel finding of this study, despite the fact that the obese rats received HFD during exercise

training, AIT not only prevented HFD-induced reduction in capillary density, but it also augmented the capillary density of sAT and mAT by approximately 25% compared to ND sedentary group.

The precise mechanism of the increase in the capillary density of AT in response to exercise training despite continued HFD feeding is not clear. Previous studies have shown that vascular endothelial growth factor (VEGF), the master regulator of AT angiogenesis, increases in response to HFD or exercise training [12,16,38]. Another possible explanation mechanism is AMP-activated protein kinase (AMPK), a key regulator of VEGF induction, activated once the cells energy charge is diminished [39]. It has been proposed that CT inhibits [8,10,40] the reduction of obesity-induced AMPK activity in AT [10,41]. So far, no studies have addressed the extent of AT AMPK activity in response to different intensities; however, research has indicated that AMPK activation in the skeletal muscle depends on the exercise intensity [42,43]. Accordingly, AIT would likely reduce energy charge more significantly in AT compared with CT, and subsequently causes an increase in the

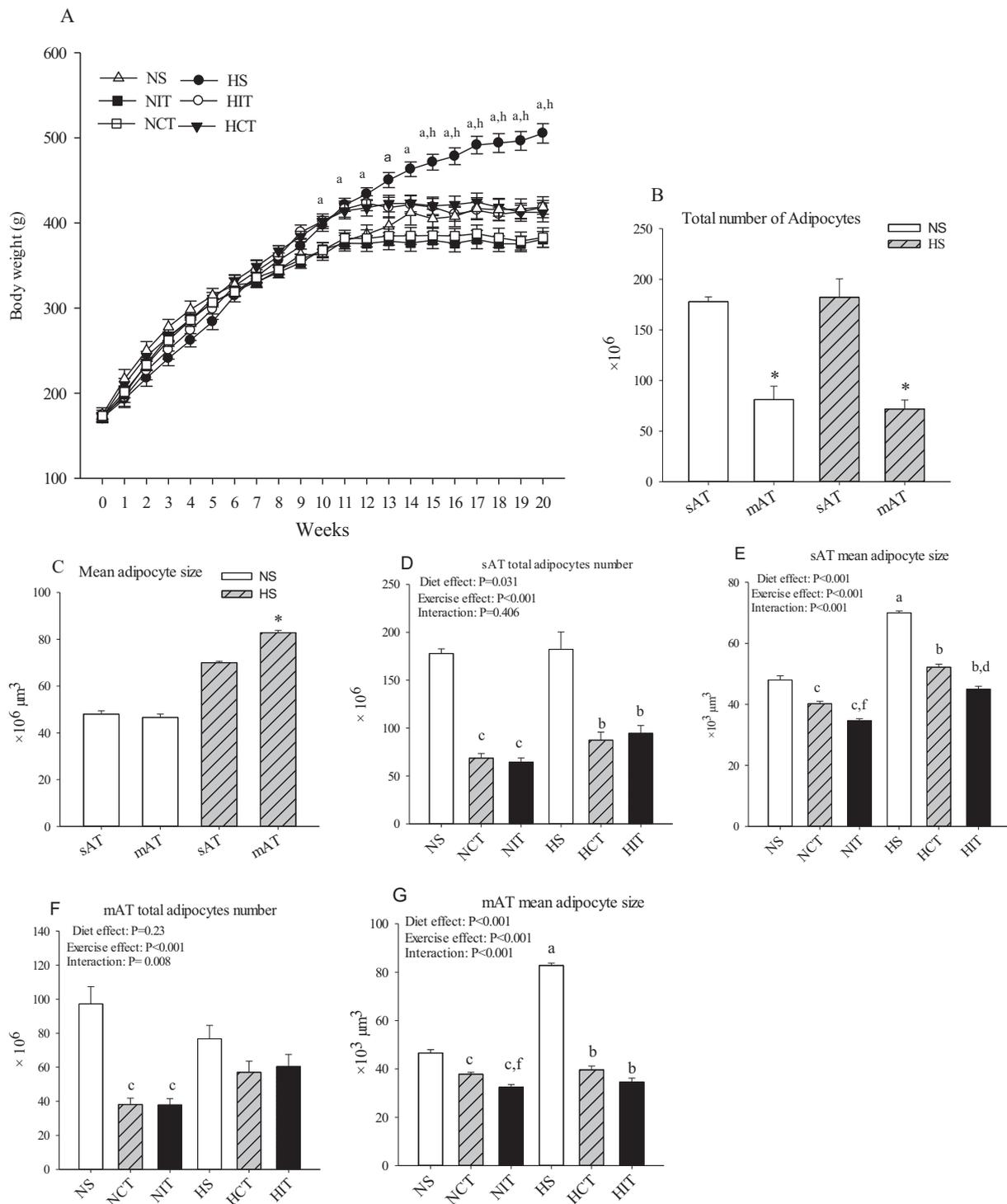


Fig. 2. Effects of diet and exercise training on adipocyte size and number in subcutaneous (sAT) and mesenteric (mAT) adipose tissue. Normal diet sedentary, NS; Normal diet plus continuous training, NCT; Normal diet plus aerobic interval training, NIT; High-fat diet sedentary, HS, High-fat diet plus continuous training, HCT; High-fat diet plus aerobic interval training, HIT; *vs. SC; ^avs. ND; ^b vs. HS; ^cvs. NS; ^d vs. HCT; ^evs. NCT; ^fvs. HCT and HIT. ($P < 0.05$). Data are represented as mean \pm SEM.

activity of AMPK, which requires further research.

Adipocytes hypertrophy-induced hypoxia is one of the most important regulators of the infiltration of immune cells into AT [37]; however, increasing the capillary density of AT and VEGF over-expression may alter the inflammatory properties of AT [5,37]. Our findings showed that the capillary density of AT was negatively correlated with M1 macrophages, yet positively associated with M2 macrophages (Table 3). Furthermore, HFD feeding switched M2 to M1

macrophages, which increased the number of M1 macrophages in mAT more than sAT, with no differences in the number of M2 macrophages between the two tissues. These results suggest that mAT is more likely to be susceptible to inflammatory responses during excessive energy intake, which might explain why a hypertrophic VAT in humans and rodents is more associated with inflammatory responses. It has been shown that adipocytes hypertrophy is accompanied by increased macrophage infiltration [44]. A possible explanation is that increasing

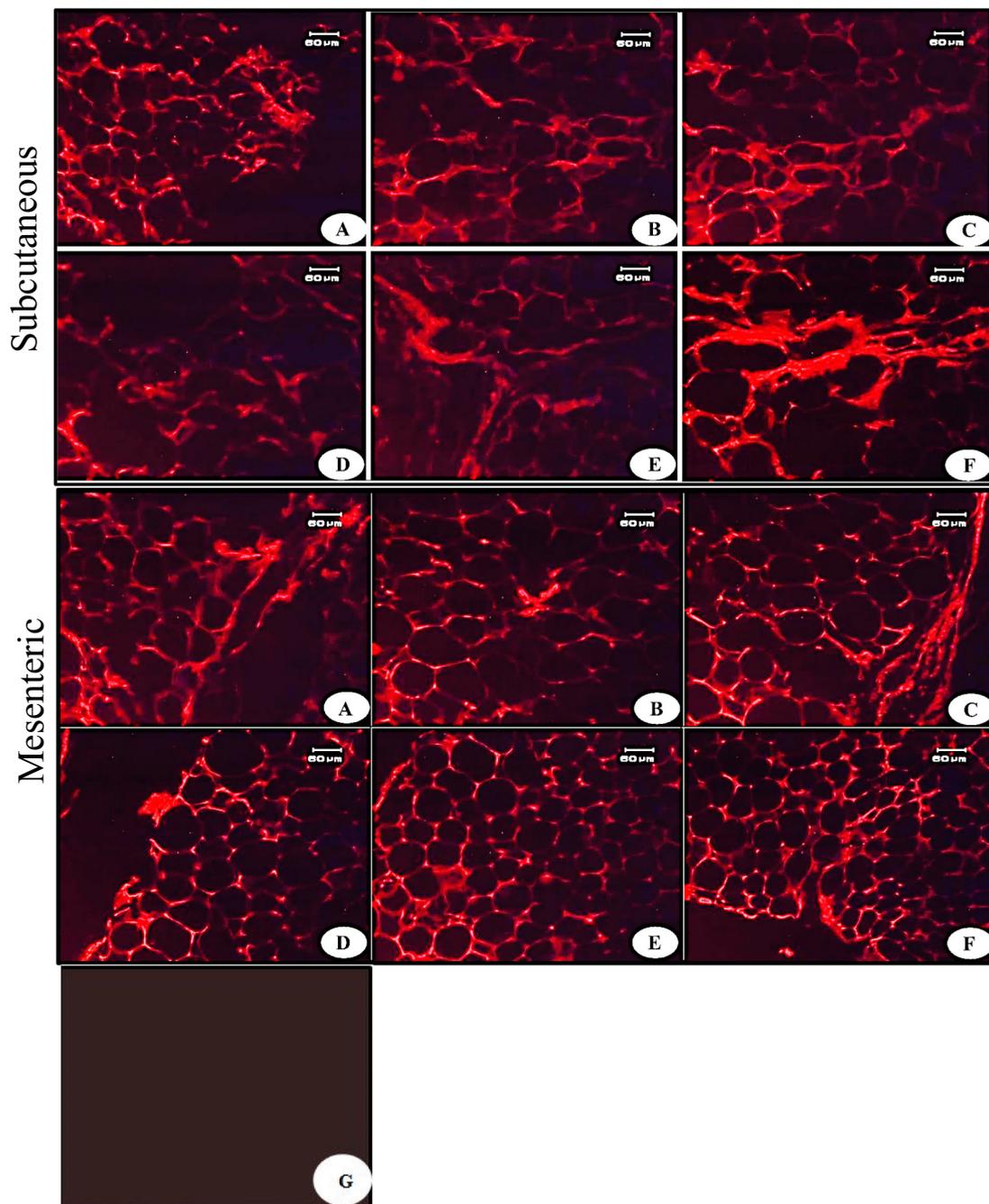


Fig. 3. Sections of subcutaneous and mesenteric adipose tissue were immune-stained with CD31-positive (red), and capillary density, number of capillaries per square millimeter (3D images) was evaluated by counting five fields in each sample (Scale bar, 60 μm). Slides were counterstained with Harris hematoxylin. A; normal diet sedentary, B; normal diet plus continuous training, C; normal diet plus aerobic interval training, D; high-fat diet sedentary, E; high-fat diet plus continuous training, F; high-fat diet plus aerobic interval training, and G: negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hypertrophic adipocytes gradually entails the death of adipocytes, which needs to be removed by M1 macrophages [2,44]. Accordingly, accumulation of higher numbers of M1 macrophages may be due to the higher mean adipocyte size in mAT.

Another important finding of this study is that HFD-induced macrophage polarization from M1 to M2 is reversed by AIT and CT in sAT and mAT, with only AIT augmenting the number of M2 macrophages in ND-fed rats, which were accompanied by higher capillary density (even in the presence of HFD). The exact mechanism for macrophages switching through exercise training is yet to be known. In agreement with our study, previous studies have shown that exercise training

promotes macrophage polarization from M1 to M2 in the AT of humans and rodents [1,15,45]. However, there have been no studies to evaluate macrophage polarization in mAT as a genuine VAT. Inconsistent with our results, Marcinko et al. (2015) reported that 6 weeks of high-intensity interval training coinciding with high-fat diet had no effects on macrophage polarization in eAT [46]. This discrepancy is probably due to the different training methods, fat depots (eAT vs. mAT), and fat mass. Here, we showed that macrophage polarization is inversely correlated with adiposity index and fat mass. Furthermore, the results indicated that the capillary density in both sAT and mAT is related to macrophage polarization, supporting previous studies [3–5]. Therefore,

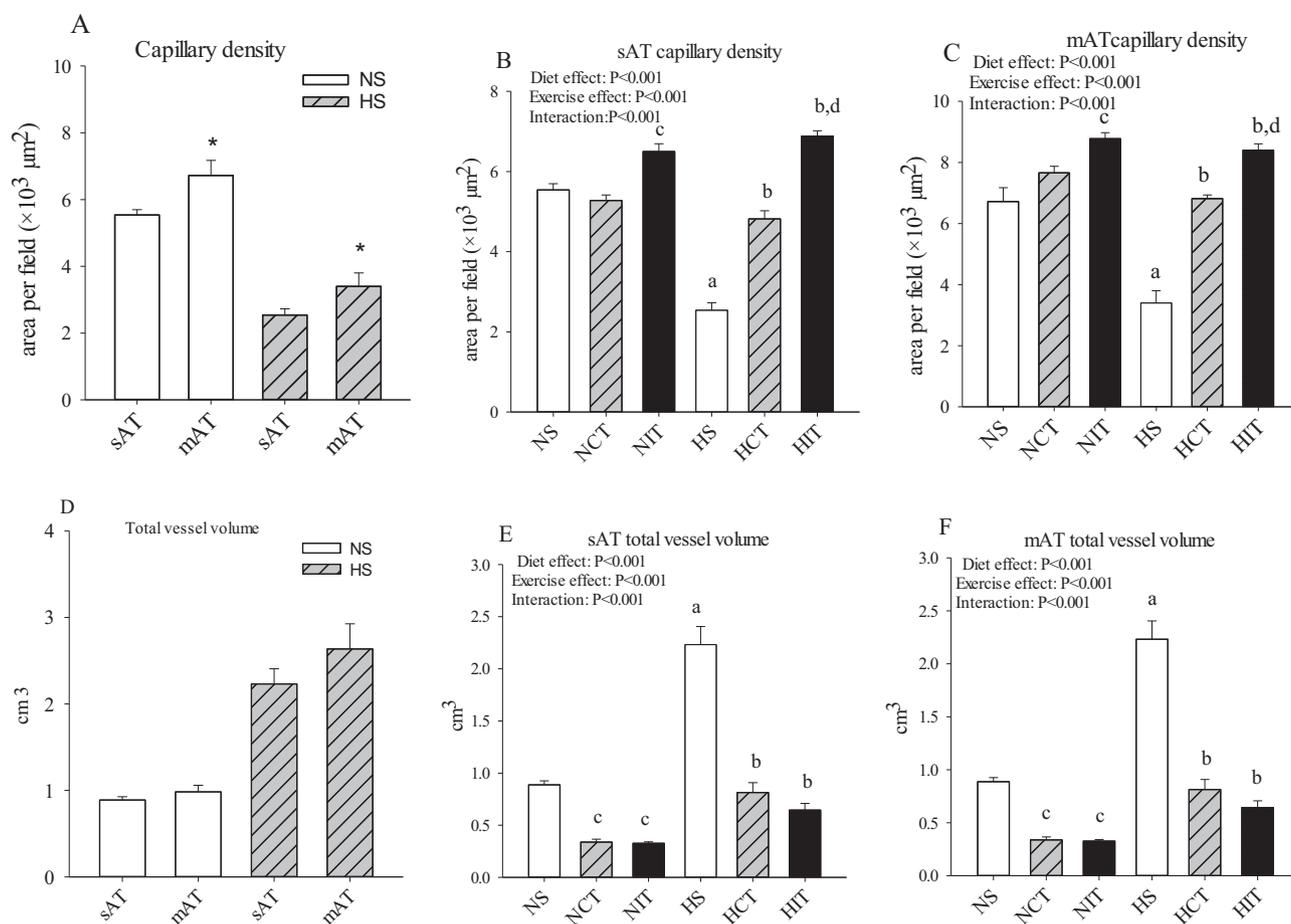


Fig. 4. Effects of diets and exercise training on capillary density and total blood vessel volume in subcutaneous (sAT) and mesenteric (mAT) adipose tissue. Normal diet sedentary, NS; normal diet plus continuous training, NCT; normal diet plus aerobic interval training, NIT; high-fat diet sedentary, HS; high-fat plus continuous training, HCT; high-fat plus aerobic interval training, HIT; *vs. SC; ^avs. ND; ^b vs. HS; ^cvs. NS; ^d vs. HCT; ($P < 0.05$). Data are represented as mean \pm SEM.

capillary density appears to be a major determinant of macrophage polarization. To confirm this assumption, we demonstrated that in the ND-fed group, only AIT significantly increased the capillary density of AT, which was associated with the augmentation in the number of M2 macrophages in both tissues. Thus, it seems that AIT and CT, in part through increasing capillary density, inhibit AT inflammation (decreasing the ratio of M1/M2) without changing the circulating immune cells. Accordingly, these results may imply that the amount of daily calorie intake is not the main factor influencing AT dysfunction, but the lack of physical activity is likely to be a major cause of metabolic disorders, AT hypoxia and inflammation.

According to previous studies, increased visceral fat accumulation is positively related to insulin resistance [17,19]. In the present study, we indicated that HFD increased plasma glucose and insulin resistance, but had no effects on plasma insulin concentration. It seems that chronic hyperglycemia impairs pancreatic beta cells sensitivity to glucose, resulting in lower insulin secretion [47].

It has been shown that insulin signaling is mainly affected by adipose tissue-derived adipokines (e.g. resistin, visfatin) [48]. Resistin, as a proinflammatory marker, is predominantly generated by visceral adipose tissue resident macrophages in human, leading to hepatic insulin resistance through the activation of protein kinase B [49]. The plasma concentration of resistin was not measured in the present study; however, macrophage polarization from M2 to M1 in mAT was significantly increased by HFD, which would likely lead to more proinflammatory cytokine expression, and insulin resistance. In this regard, it has been shown that decreasing M2 macrophages and increasing M1 macrophages in adipose tissue promote insulin resistance [50]. On the other

hand, despite the high-fat diet intake, HFD-induced insulin resistance was reversed by AIT and CT. In agreement with our study, Higa et al. (2014) reported that high-intensity interval training reduced cafeteria-diet-induced hyperglycemia and insulin resistance [10] which can be due to the increase in insulin signaling and action in AT [7]. To explain these results, our data demonstrated that the augmentation in the mean adipocyte size, reduction in capillary density, and macrophage switching from M2 to M1 are associated with systemic insulin resistance; however, the results suggest that despite high-fat diet feeding, AIT and CT may prevent insulin resistance at least in part through reducing the mean adipocyte size, and increasing the capillary density, and the anti-inflammatory properties of mAT and sAT during obesity.

Our findings need to be confirmed by other technical approaches such as western blot or flow cytometry, which is one of the limitations of the present research. Another limitation was our inability to investigate the key elements involved in angiogenesis signaling pathways (e.g. VEGF) and macrophage polarization (e.g. the cyclic AMP-responsive element binding) to specify the exact mechanism for the increase in capillary density and macrophage polarization of adipose tissue in response to diet and exercise training. Finally, we were unable to analyze eAT, although the results suggest that the physiological response of mAT in rodents resembles VAT's in human to diet or exercise training; accordingly, future studies should provide further insight into the similarities and differences between mAT and eAT in rodents with VAT in humans.

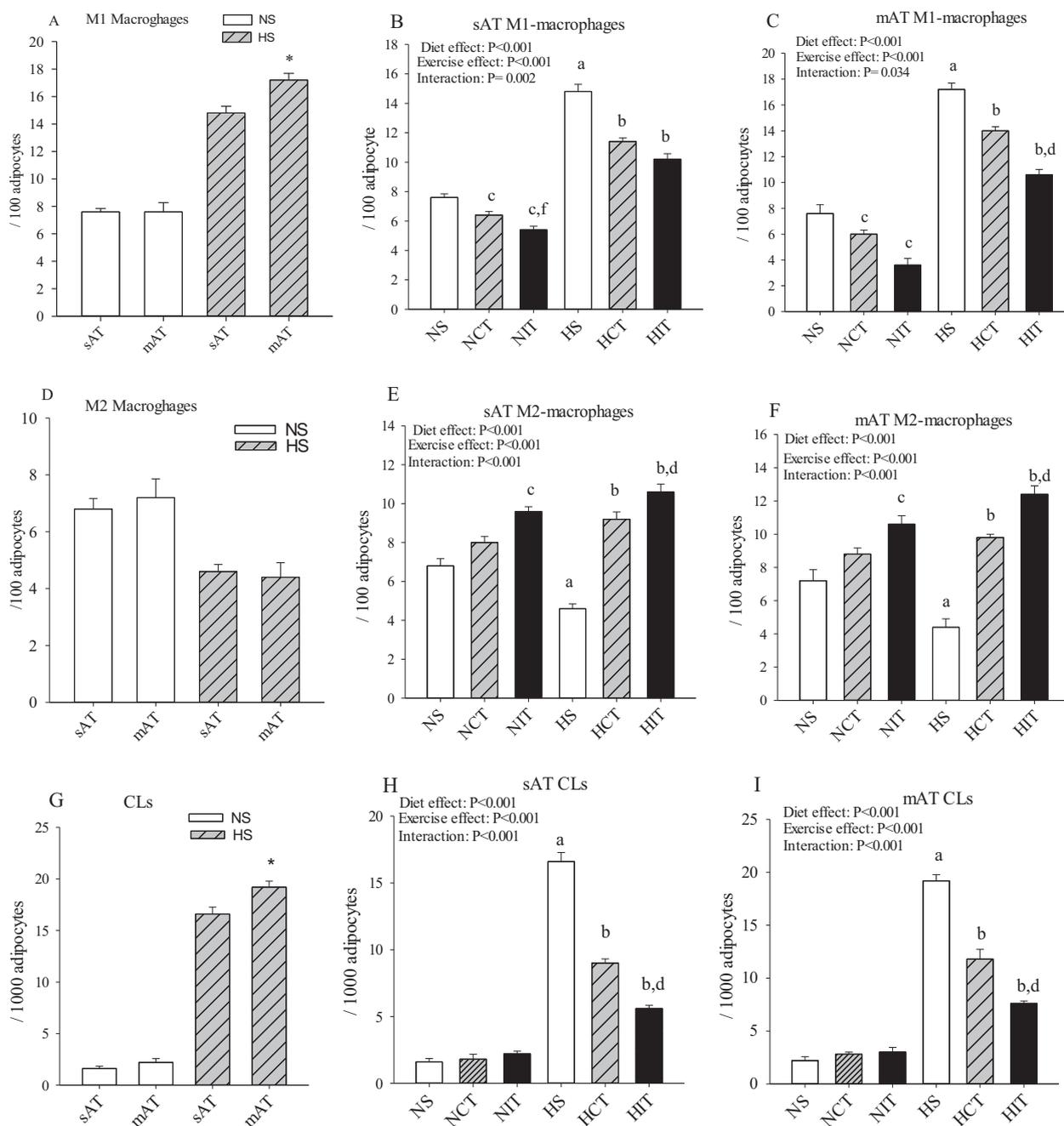


Fig. 5. Effects of diets and exercise training on macrophage polarization (M1 and M2) and crown-like structure (CLs) in subcutaneous (sAT) and mesenteric (mAT) adipose tissue. Normal diet sedentary NS; normal diet plus continuous training, NCT; normal diet plus aerobic interval training, NIT; high-fat diet sedentary, HS; high-fat plus continuous training, HCT; high-fat plus aerobic interval training, HIT; *vs. SC; ^a vs. ND; ^b vs. HS; ^c vs. NS; ^d vs. HCT; ^f vs. NCT. ($P < 0.05$). Data are represented as mean \pm SEM.

5. Conclusion

The current study provides novel and important insights into the mesenteric and subcutaneous adipose tissues remodeling; for instance, adipose tissue expansion is likely occurred by adipocytes hypertrophy rather than hyperplasia, which is more pronounced in the mesenteric adipose tissue. We further showed that AIT is a good exercise training strategy for increasing the capillary density along with anti-inflammatory macrophages in adipose tissue of obese and normal weight subjects, thereby ameliorating insulin resistance. Finally, we provided data on the critical role of capillary density in macrophage polarization and expansion of mesenteric adipose tissue, which is to be considered in the design of future intervention research.

Authors contributions

S.K., E.T.G., and A.S., conceived and designed the experiments; S.K, G.H performed the experiments; S.K, E.T.G, and G.H, contributed reagents/materials/analysis tools; S.K., data analysis; S.K, writing original draft, review and editing. All authors read and approved the final manuscript.

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Table 3
Simple correlation (Pearson r) between data variables in the both adipose tissues.

	Subcutaneous			Mesenteric		
	N	MAS	Capillary density	N	MAS	Capillary density
Body weight	0.581**	0.827**	−0.698**	0.462*	0.777**	−0.777*
Adiposity index	0.778**	0.858**	−0.669**	0.613**	0.822**	−0.809**
CD86	0.503**	0.909**	−0.711**	0.316	0.702**	−0.760**
CD206	−0.723**	−0.708**	0.820**	−0.400*	−0.805**	0.793*
CLs	0.433*	0.894**	−0.781**	0.230	0.772**	−0.753**
Insulin	0.612**	0.570**	−0.548**	0.445*	0.648**	−0.561**
Glucose	0.628**	0.839**	−0.592**	0.536**	0.677**	−0.722**
HOMA-IR	0.649**	0.665**	−0.526**	0.468**	0.725**	−0.653**
HDL	0.131	0.009	−0.120	0.161	0.087	−0.097
TG	0.469**	0.660**	−0.356	0.369*	0.489**	−0.502**
LDL	0.375*	0.276	−0.235	0.220	0.349	−0.257
TC	0.478**	0.509**	−0.458*	0.205	0.588**	−0.550**

r values significant set at ** $P < 0.01$, * $P < 0.05$. Adipocytes numbers, N; Mean Adipocyte size, MAS.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2019.01.037>.

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