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Female athlete triad affects rat intestinal morphology and sucrase-isomaltase expression

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Abstract

Female athletes follow a strict diet and perform rigorous exercise to boost their performance, which induces health issues called the female athlete triad (FAT), defined as the combination of disordered eating, amenorrhoea and low bone mineral density. It is known to have a significant effect on bones. However, its effects on the small intestine, which is responsible for nutrient uptake into the body, remain unclear. In this study, we created an animal model of FAT to examine its effects on digestive and absorptive molecules in the small intestine. Thirty 5-week-old female Sprague-Dawley (SD) rats with an initial body weight of about 147 g were divided into control (Con, $n = 7$), exercise (Ex, $n = 7$), food restriction (FR, $n = 8$) and exercise plus food restriction (FAT, $n = 8$) groups. The rats were subjected to 4 weeks of wheel running (Ex, FAT) and 50–40 % food restriction (FR, FAT) to examine the effects on bone and typical digestive enzymes and transporters in the jejunum. Two-way ANOVA and the Kruskal–Wallis test were used for statistical analysis of normal and non-normal data, respectively. Four weeks of exercise and food restriction decreased bone weight (*vs.* other group $P < 0{\cdot}01$) and bone breaking power (*vs.* other group $P < 0{\cdot}01$). Villus height decreased in the jejunum (*vs.* other group $P < 0{\cdot}01$), but the expression of typical macronutrients digestive enzyme and absorptive molecules remained unchanged. In contrast, sucrase-isomaltase gene (*v.* Ex $P = 0{\cdot}02$) and protein expression were increased (*vs.* other group $P < 0{\cdot}05$). The study findings show that FAT affects sucrase-isomaltase without histone methylation changes.

Key words: **Female athlete triad (FAT): Bone: Intestine: Female athlete**

Athletes are central to various sports, and in recent years, the number of female athletes has increased, and their performance has improved remarkably. Alongside this, health issues in female athletes have become apparent[1].

In case of weight-sensitive sports, such as those involving endurance, aesthetics and different weight categories, body weight and composition affect athletic performance[2,3]. Therefore, athletes often undertake extreme methods of weight loss, such as restricting food intake and excessive training[2], which causes serious health issues such as the female athlete triad (FAT), defined as the combination of disordered eating, amenorrhoea and low bone mineral density (BMD)[4]. Moreover, inadequate energy and nutrient uptake during the growth period induces delayed growth and development during puberty[5]. Although exercise confers various benefits to the body, a prolonged period of low availability of energy due to restrictions on food intake and excessive exercise is known to reduce bone strength both directly and indirectly. Indirectly, this happens because of oestrogen deficiency owing to menstrual abnormalities. Whereas, the abnormal secretion of metabolic hormones is a direct cause for the reduction in bone strength[6].

Several effects of FAT on bone have been reported, as well as on hormone secretion in adipocytes[7], thyroid, pancreas and ovaries[8–10]. Therefore, FAT is expected to have some detrimental effects on various organs of the body. FAT is often attributed to excessive exercise and strict dietary restrictions, which are known to have adverse effects on the intestinal tract. Excessive exercise is known to damage the intestinal tract and cause leaky gut[11], and gene expression of enzymes and transporters involved in digestion and absorption is markedly reduced when

Abbreviations: BMD, bone mineral density; ChIP, chromatin immunoprecipitation; FAT, female athlete triad; FR, food restriction; qPCR, quantitative PCR; SI, sucrase-isomaltase.

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dietary nutrients no longer stimulate the intestinal epithelial cells, such as in fasting[12]. Although the small intestine is known for its nutrient uptake, barrier function and secretion of various hormones[13–15], FAT may have a severe negative impact on the intestinal tract, which performs important functions. Notably, nutrient uptake is important for the smooth progression of biological activities in the body and athletes must therefore consider the effects of excessive exercise and severe dietary restrictions on the small intestine. Therefore, in this study, we aimed to develop an animal model of FAT to characterise the morphology of the small intestine, particularly the jejunum, and changes in molecules related to nutrient uptake.

Materials and methods

Animal treatments

This study was conducted in accordance with the principles and guidelines for international animal care and approved by the Animal Experimental Committee of the University of Tsukuba (approval number 21-1007).

Thirty 5-week-old female Sprague-Dawley (SD) rats (Japan CLEA Co., Ltd.) were used in this experiment. After acclimatisation to the environment for 1 week, the animals were divided into four groups: no exercise and ad libitum food intake (Con, *n* 7), exercise and ad libitum food intake (Ex, *n* 7), no exercise and 50–60 % food restriction (FR, *n* 8) and exercise and 50–60 % food restriction group (FAT, *n* 8). The minimum sample size was estimated and determined from our unpublished data. The experimental period was 4 weeks. During the study period, the FR and FAT groups were fed 50 % of the food intake of the Ex group for the first week. However, because of the rapid weight loss observed during the first week (approximately 20 % weight loss in the FAT group), the FR and FAT groups were fed 60 % of the food intake of the Ex group from the second week. Since FAT is primarily attributed to reduced available energy, this study limited only energy sources, not vitamins and minerals. The composition of the diet is listed in Table 1. During the experiment, the rats had free access to the rotating wheel and exercised spontaneously. The rats were kept in a stainless-steel wire case (13 × 13 × 22 cm) for the Con and FR groups and in a cage with a rotary wheel (1 m per lap, cage size: 35 cm high–27 cm wide–35 cm deep) for the Ex and FAT groups to perform the exercise. The room temperature and humidity were maintained at 25 ± 1°C and 80 ± 10 % under a constant 12:12 h light–dark cycle (light 6:00–18:00).

Daily data and sample collection

The body weight, food intake and running distance were recorded daily. The rats were made to fast for 2 h prior to autopsy and isoflurane (Mylan Inc.) was inhaled. After confirming the disappearance of the rise reflex, whole blood was quickly drawn from the abdominal aorta and the rats were killed. The blood was kept on ice for 30 min and then centrifuged (2500 rpm, 4°C, 15 min) to obtain serum. After killing, the femur and lumbar spine were collected, and the lumbar spines were preserved in 70 % ethanol. The femurs were collected, and the wet weight was measured. The jejunum was collected as previously described[12]. Briefly, the small intestine was cut in half and the duodenum side was used as the jejunum. The central parts of the jejunum (approximately 1 cm) were used for haematoxylin and eosin staining. The remaining sample was cut open longitudinally, the muscle layer was removed and the mucosa was collected. Femurs were stored at 4°C until biomechanical testing.

Measurement of bone strength

The fracture force and fracture energy were measured using a bone fracture characteristic-measuring device (DYN-1255, Iio Electric Co.) using the femurs. The conditions were as follows: distance between fulcrums, 1 cm; plunger speed, 100 m/min; full scale, 50 kg and chart speed, 120 cm/min.

Measurement of bone mineral content and bone mineral density

Bone mineral content and BMD of the L3–L6 lumbar spine were measured using dual-energy X-ray absorptiometry (DXA; QDR-4500A, Hologic Inc.). All scans were performed using small animal mode and high regional resolution.

Haematoxylin and eosin staining and measurements

Jejunum samples were stained with haematoxylin and eosin according to protocols from previous studies[12,16]. Mucosal thickness, villus height, crypt depth and villus width were measured in at least five villi/rat as previously described[17]. Briefly, crypt depth was measured from the lowest layer of the mucosa excluding the muscular layer to the sub-basal portion of the villus, and villus height was measured from the sub-basal to apical portion of the villus, and the sum of these two was used as the mucosa thickness. Villus width was measured at the thickest part of the villus.

All images were captured using a microscope (BZ-X710; Keyence).

Immunoblotting

All immunoblotting procedures were performed according to a previous study[12]. Briefly, total protein was extracted from jejunal mucosa samples using a lysis buffer (1 % NP-40, 50 mM Tris, 150 mM NaCl, 1 mM EDTA including proteinase inhibitor tablets (cOmpleteTM mini, Roche)). Protein concentration was measured using a Takara BCA Assay kit (T9300A, Takara Bio) and microplate reader (Varioskan LUX, Thermo Fisher Scientific). Finally, the protein samples were adjusted to a concentration of 1 μg/μl. The total protein (15 μg/lane) was used for gel electrophoresis. For western blotting, primary antibodies against liver-type fatty acid-binding protein (1:1000, sc-374537, Santa Cruz Biotechnology), intestinal-type fatty acid-binding protein (1:1000, sc-374482, Santa Cruz Biotechnology), APOA1 (1:500, sc-58230, Santa Cruz Biotechnology), alanyl aminopeptidase (1:1000, sc-13536, Santa Cruz Biotechnology), SLC15A1 (1:1000, sc-373742, Santa Cruz Biotechnology), SGLT1 (1:1000, A11976, ABclonal Technology), GLUT2 (1:1000, 20436-1-AP, Proteintech Group Inc.) and sucrase-isomaltase (1:1000, sc-393470, Santa

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Table 1. Composition of daily diet of rats used in the study

Materials	Normal diet % (0·6 % Ca, 0·6 % P)	Food restriction (50 % FR) % (1·2 % Ca, 1·2 % P)	Food restriction (60 % FR) % (1·0 % Ca, 1·0 % P)
Glucose monohydrate	62·15	55·15	57·56
Casein*	18	18	18
Cystine	0·2	0·2	0·2
Cottonseed oil	10	10	10
$CaCO_3$	1·48	3·01	2·51
KH_2PO_4	1·27	2·76	2·21
K_2HPO_4	1·62	3·53	2·83
Roughage	3	3	3
Choline chloride	0·2	0·2	0·2
Water-soluble vitamin mixture†	0·1	0·2	0·17
Oil-soluble vitamin mixture‡			
Ca·P-free salt mixture§	2	4	3·33
Energy (kj/100 g)	1610·8	1460·2	357

* Casein contained 45 mg Ca/100 g and 127 mg P/100 g.
† Components of the water-soluble vitamin mixture (%): thiamine, 0·5; riboflavin, 0·5; pyridoxine, 0·5; calcium pantothenate, 2·8; nicotinamide, 2·0; inositol, 20·0; folic acid, 0·02; vitamin B_{12}, 0·002; biotin, 0·01 and glucose monohydrate, 73·7.
‡ The rats received a supplement of the following oil-soluble vitamins in cottonseed oil/week: β-carotene, 70 μg; 2-methyl-1·4-naphthoquinone, 105 μg; α-tocopherol, 875 μg and vitamin D_3, 525 mg.
§ Ca- and P-free salt mixture (%): KCl, 57·7; NaCl, 20·9; $MgSO_4$, 17·9; $FeSO_4 \cdot 7H_2O$, 3·22; $CuSO_4 \cdot 5H_2O$, 0·078; NaF, 0·133; $CoCl_2 \cdot 6H_2O$, 0·004; KI, 0·01; $MnSO_4 \cdot 5H_2O$, 0·06; $ZnSO_4 \cdot 7H_2O$, 0·44; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0·005.

Cruz Biotechnology) were used. Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, #7074) and anti-mouse IgG (Cell Signaling Technology, #7076) were used as secondary antibodies. Signals were detected using chemiluminescence reagent (EzWestLumi One or EzWestLumi plus, ATTO). Blots were scanned using a chemiluminescence imaging system (FUSION FX7.EDGE; Vilber Lourmat).

Quantitative PCR

All quantitative PCR (qPCR) procedures were performed as previously described[12]. Briefly, total RNA was extracted from the jejunal mucosa using Sepasol ®-RNA I Super G (Nacalai Tesque). Concentration and quality of RNA were measured using a spectrophotometer (NanoDrop OneC, Thermo Fisher Scientific). RNA quality was assessed with the 260/280 nm and 260/230 nm absorbance ratios (>1·8). Total RNA samples were adjusted to 400 ng/tube, and reverse transcription was performed using 5 × PrimeScript RT Master Mix (RR 036 A, Takara Bio) in a thermal cycler (TP 350, Takara Bio). After reverse transcription, TB Green ® Premix Ex Taq ™ II (Takara Bio) was used to perform qPCR using QuantStudio® 5 (Thermo Fisher Scientific). qPCR was performed with the following conditions: Hold stage: 95°C for 20 s, PCR stage: 95°C for 1 s, 60°C for 20 s, 40 cycles and Melt curve stage: 95°C for 1 s, 60°C for 20 s, 95°C for 1 s. The 28S ribosomal RNA was used as the housekeeping gene. The primer sequences were adopted from a previous study[16]. The primer sequences for the 28S ribosomal RNA were as follows: forward 5′-CCCAGAAAAGGTGTTGGTTGA-3′ and reverse 5′-TGATTCGGCAGGTGAGTTGTT-3′. The primer sequences for sucrase-isomaltase (Si) were as follows: Forward 5′-GCAGAAGGCTACATGGA-3′ and Reverse 5′–3′ and Reverse 5′-CCTTGCGACTGTCTCA-3′. The quantification cycle (Cq) value of the target gene was normalised to that of the housekeeping gene. The results were analysed using the $2^{-\Delta\Delta Ct}$ method adapted from Livak and Schmittgen[18] and expressed relative to the levels in the Con group.

Sucrase activity assay

Sucrase activity assay was conducted as previously described[19,20]. Each jejunal mucosa sample was homogenised in buffer containing 10 mM KH_2PO_4 and 10 mM K_2HPO_4 (pH 7·0). Mixtures of the homogenate and sucrose were incubated at 37°C for 20 min. After incubation, the reaction temperature was increased to 100°C for 2 min to inactivate the sucrase. To detect the hydrolysed glucose from sucrose, Tris-HCl-glucose oxidase reagent (3:7 ratio of 0·5 M Tris-HCl, pH 7·0 to Glu CII test Wako was added to each well and subsequently incubated at 37°C for 30 min. Then, 200 μl of the reaction solution was transferred to a new plate, and the absorbance was measured at 505 nm. The total protein concentration of each homogenate was determined using a bicinchoninic acid (BCA) protein assay kit (Takara Bio). One unit of sucrase activity corresponds to 1 mmol of glucose produced/min. It was also standardised by protein concentration of the homogenate.

Chromatin immunoprecipitation quantitative PCR

Chromatin immunoprecipitation (ChIP) qPCR was performed as previously described with minor modifications[21,22]. The mucosa removed from the jejunum was fixed in formaldehyde solution (1 % formaldehyde, 4·5 mM HEPES (pH 8·0), 9 mM NaCl, 0·09 mM EDTA, 0·04 mM ethylene glycol tetraacetic acid) in PBS for 30 min at 37°C. The reaction was terminated with 2 M glycine (final concentration, 150 mM). After washing in fluorescence-activated cell sorter solution (1 × PBS(-), 2 % bovine serum, 0·05 % NaN3), the samples were sonicated in sodium dodecyl sulfate lysis buffer (50 mM Tris-hydrochloride (pH 8·0), 10 mM EDTA (pH 8·0), 1 % sodium dodecyl sulfate) and

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ChIP dilution buffer (50 mM Tris–HCl (pH 8·0), 167 mM NaCl, 1·1 % Triton X-100, 0·11 % sodium deoxycholate) (ratio 1:4) with a protease inhibitor cocktail (Wako) to obtain 200- to 500-bp DNA fragments. The ChIP assay was performed using the following antibodies: anti-trimethyl-histone H3K4 (1:160, A2357, ABclonal) or 1 mg of control rabbit (Ig)G. The precipitated DNA was analysed by real-time PCR. ChIP results were expressed as a percentage of the PCR signal for input DNA using the delta–delta method[18], The Cq value was calculated by raising 2 to the power of $Cq_{\text{immunoprecipitated (IP) DNA}}$-$Cq_{\text{input DNA}}$ ($100 \times [2^{(\text{Cq of Input-Cq of IP sample})}]$). PCR equipment and conditions used were the same as in the qPCR section. Optimised PCR primer sequences and gene regions were obtained as previously described[22].

Statistical analysis

Data are shown as mean ± SEM. Two-way ANOVA was conducted. If interaction was observed, followed by Tukey's post hoc test was used to compare each group (independent variables: exercise (Ex) and food restriction (FR)). In qPCR, data were not normally distributed; therefore, the Kruskal–Wallis H test, followed by a two-stage Benjamini, Krieger and Yekutieli false discovery rate procedure as a post hoc test, was used. An unpaired *t*-test was used for comparison between the two groups. Statistical significance was set at $P < 0·05$ and significance tendency was set at $P < 0·1$. Statistical analyses were performed using the GraphPad Prism 8.4.3 software for Mac.

Results

Combination of continuous food restriction and exercise inhibited rat growth

There was a significant interaction between exercise and food restriction on final body weight, body weight gain, food intake and food efficiency. Final body weight in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P < 0·0001$; *vs.* Ex $P < 0·0001$; *vs.* FR $P = 0·0002$). Body weight gain in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P < 0·0001$; *vs.* Ex $P < 0·0001$; *vs.* FR $P = 0·0003$). Food intake in FAT was lower compared with those in Con and Ex groups (*vs.* Con $P < 0·0001$; *vs.* Ex $P < 0·0001$). Food intake in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P = 0·0001$; *vs.* Ex $P < 0·0001$; *vs.* FR $P = 0·0007$). The FAT group had a significantly higher average running distance than the Ex group. In addition, although the difference was not significant, the blood glucose levels in the FAT group were lower than those in the Con and Ex groups ($P < 0·104$).

Combination of continuous food restriction and exercise negatively affected bone parameters

There was a significant interaction between exercise and food restriction on bone weight, breaking power and mineral density. Bone weight in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P = 0·0008$; *vs.* Ex $P < 0·0001$; *vs.* FR $P = 0·0129$). Bone breaking power in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P = 0·0046$; *vs.* Ex $P = 0·0015$; *vs.* FR $P = 0·0358$). Food intake in FAT was lower compared with those in Con and Ex groups (*vs.* Con $P = 0·0057$; *vs.* Ex $P = 0·0019$). The main effect of food restriction was observed in bone breaking and mineral content, but the interaction was not observed.

Combination of continuous food restriction and exercise atrophied the jejunal villus

There was a significant interaction between exercise and food restriction on mucosal thickness, villus height and crypt depth. Mucosal thickness in FAT was lower compared with those in Con, Ex and FR groups (*vs.* Con $P = 0·0023$; *vs.* Ex $P < 0·0001$; *vs.* FR $P < 0·0001$). Villus height in FAT was lower compared with Ex group ($P < 0·0001$). The main effect of Ex was observed in villus width, but the interaction was not observed.

Digestive enzymes and transporters of lipid, protein and glucose were unchanged

There were no significant differences in the protein expression of liver-type fatty acid-binding protein, intestinal-type fatty acid-binding protein and apolipoprotein A1 (APOA1), which are typical lipid transporters.

The main effect of food restriction was observed in protein expression of alanyl aminopeptidase ($P = 0·0180$) and solute carrier family 15 member 1 (SLC15A1) ($P = 0·0171$), which are typical digestive enzymes and protein transporters, but the interaction was not observed.

There were no significant differences in the protein expression of sodium-glucose transporter 1 (SGLT1). The main effect of exercise and food restriction was observed in glucose transporter 2 (GLUT2) (Ex $P = 0·0208$; FR $P = 0·0049$), which are typical glucose transporters, but the interaction was not observed.

Combination of continuous food restriction and exercise increased sucrase-isomaltase expression and sucrase enzyme activity

Gene expression of *Si* was significantly increased in the FAT group compared with that in the Ex group ($P < 0·05$ *vs.* Ex). There was a significant interaction between exercise and food restriction on the protein expression of SI. SI in FAT was higher compared with those in Con, Ex and FR groups (*vs.* Con $P = 0·0066$; *vs.* Ex $P < 0·0048$; *vs.* FR $P < 0·0245$). The main effects of food restriction were observed in sucrase enzyme activity, but the interaction was not observed.

Combination of continuous food restriction and exercise did not affect the epigenome of Si

There was no increase in histone H3K4 trimethylation in any region of the *Si* gene in any group.

Discussion

In this study, we developed an animal model of FAT and examined the histological and molecular characteristics of the jejunum in this rat model. As a result, we revealed the following

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Table 2. The effect of continuous food restriction and exercise on basic parameters

	Control (Con)		Exercise (Ex)		Food restriction (FR)		Ex+FR (FAT)		Ex	FR	Interaction
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P*-value	*P*-value	*P*-value
Initial body weight (g)	147·4	2·68	147·43	2·71	147·38	3·02	147·25	3·52	0·9801	0·8333	0·9804
Final body weight (g)	244·14	3·32^{a}	262·71	6·09^{b}	172·13	1·72^{c}	119·88	3·65^{d}	0·0032	<0·0001	<0·0001
Body weight gain (g/d)	3·2	0·06^{a}	3·8	0·12^{b}	0·8	0·11^{c}	-0·9	0·17^{d}	0·0042	<0·0001	<0·0001
Food intake (g/d)	16·12	0·19^{a}	20·59	0·62^{b}	8·83	0·00^{c}	8·79	0·01^{c}	<0·0001	<0·0001	<0·0001
Food efficiency (g/d)	0·20	0·00^{a}	0·18	0·00^{a}	0·09	0·01^{b}	-0·09	0·02^{c}	0·0003	<0·0001	0·0009
Running distance (km/d)	–		8·78	1·57	–		16·92	2·57**	–	–	–
Blood glucose level (mg/dl)†	143·6	10·9	151·4	12·3	152·4	14·5	102·2	9·4‡,§	0·0292	0·0689	0·0550

† $n=7$ in the FAT group because blood could not be collected in one individual. In the FAT group, the final body weight, body weight gain and food efficiency were significantly lower than those in the other three groups. In addition, although the difference was not significant, the blood glucose levels in the FAT group tended to be lower than those in the Con and Ex groups.
‡ $P=0·104$ *vs.* Con.
§ $P=0·083$ *vs.* Ex.Con ($n=7$), Ex ($n=7$), FR ($n=8$), FAT ($n=8$). FAT, female athlete triad.
All data are presented mean ± SEM.
Different letters indicate significant differences.
Asterisks indicate significant differences.

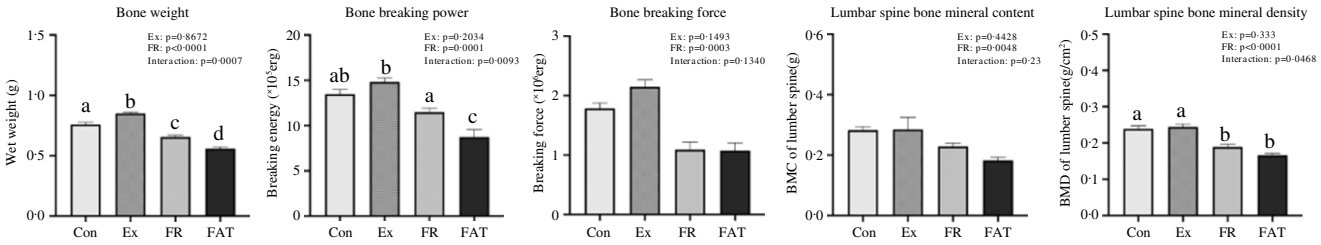

Fig. 1. The effect of continuous food restriction and exercise on parameters of bone. All data are presented as mean ± SEM. Different letters indicate significant differences. Con: control ($n=7$), Ex: exercise ($n=7$), FR: food restriction ($n=8$), FAT; Ex+FR ($n=8$). Lumbar spine bone mineral content and density in the FAT group used $n=7$ because they could not be measured in one individual. Bone weight and breaking power were significantly lower in the FAT group than in the other three groups. FAT, female athlete triad.

characteristics of the jejunum: (1) atrophy of the jejunal villus; (2) no change in typical digestive enzyme and transporters of lipid, protein and (3) increased SI gene and protein expression with no histone H3K4 methylation changes.

Previous studies have adopted either food restriction at 70 % of normal feeding for 13 weeks[23] or wheel running for 12 or 14 weeks, in addition to food restriction[24,25], to develop the FAT model. These models showed significantly reduced weight gain and caused a significant decrease in BMD. In accordance with the results of previous studies, in our model, BMD in the FAT group was significantly lower than that in the Con group, and body weight, bone weight and bone breaking power were also significantly lower than in the other groups (Table 2, Fig. 1). In addition, there was a significant increase in running distance (Table 2), suggesting a loss in the running cycle and a disruption in the sexual cycle as in previous studies[24,26]. Therefore, we obtained a FAT-like phenotype in growing rats with growth inhibition and reduced bone strength.

Jejunal morphological features were confirmed by HE staining. The results showed that all parameters, except villus thickness, were significantly increased in the Ex group relative to the Con group, whereas the effect was counteracted in the FAT group. In particular, the mucosal thickness and villus height were significantly lower than those in the other three groups (Fig. 2). Some studies have focussed on the effects of exercise on the alteration of villus morphology in the small intestine. Shin et al.[27] reported that 4 weeks of treadmill running increased the expression of tight junction proteins in old mice. In addition, Carbajo-Pescador *et al.* reported that combined aerobic and resistance training corrects the decrease in intestinal tight junction mRNA and villus overgrowth in rats fed a high-fat diet[28]. On the other hand, Costa *et al.* reported that increased exercise duration and intensity causes endotoxemia, with intestinal damage and intestinal permeability and malabsorption[29]. Thus, exercise is expected to have a positive effect on the morphology and function of the intestine, but excessive intensity and duration are suggested to have a negative effect. Similarly, food restriction has also been reported to have a positive effect on the intestine as an acute response[30], but prolonged restriction (e.g., total parenteral nutrition, fasting, malnutrition) has an adverse effect[19]. In the FAT group, the imbalance between energy demand and supply in the body may have had a negative effect on intestinal morphology. Indeed, jejunal biopsies of malnourished children showed significant jejunal villus atrophy and increased intestinal permeability compared to those with normal energy sufficiency[31]. Thus, villus atrophy may have occurred in a state of imbalance between energy demand and supply.

The expression levels of typical digestion and absorption molecules of the three major nutrients were measured. Unexpectedly, there was no change in the expression of digestive enzymes or transporters of lipid, protein and glucose (Figs. 3 and 4). Previous studies have reported that digestion and

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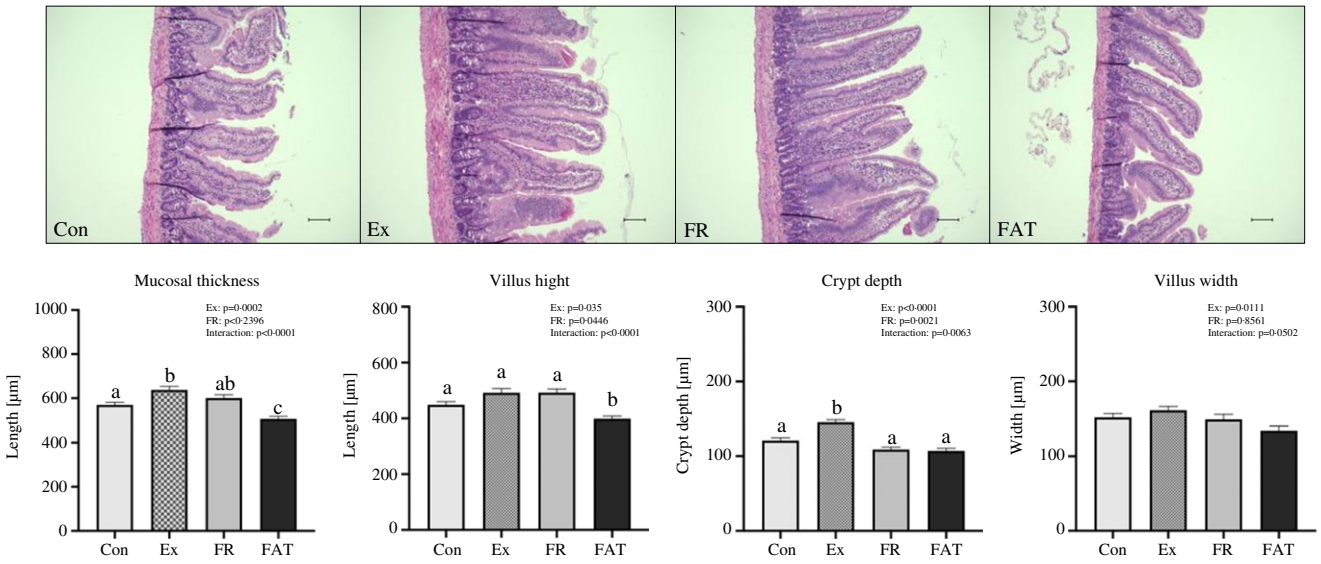


Fig. 2. The effect of continuous food restriction and exercise on jejunum morphology. Images were obtained by optical microscope (100× magnification). At least 5 villi/rat were measured. Black bar indicates 50 μm. All data are presented as mean ± SEM. Different letters indicate significant differences. Con: control ($n=7$), Ex: exercise ($n=7$), FR: food restriction ($n=8$), FAT; Ex+FR ($n=8$). Mucosal thickness and villus height were significantly decreased in the FAT group compared with the other three groups. FAT, female athlete triad.

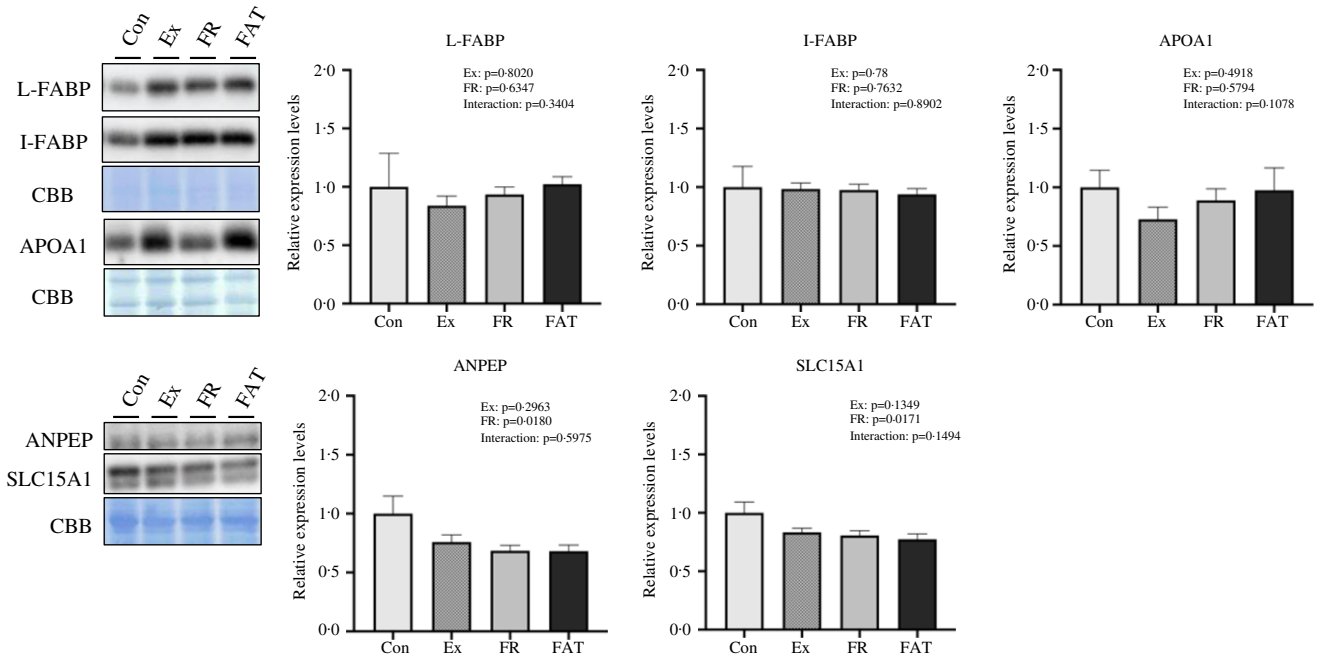


Fig. 3. The effect of continuous food restriction and exercise on the expression levels of digestive enzymes and lipid and protein transporters. Blotting images show representative images. All data are presented as mean ± SEM. L-FABP: liver type fatty acid binding protein, I-FABP: intestinal type fatty acid binding protein, APOA1: apolipoprotein A1, ANPEP: alanyl aminopeptidase, SLC15A1: solute carrier family 15 member 1, CBB: Coomassie brilliant blue stain. Con: control ($n=7$), Ex: exercise ($n=7$), FR: food restriction ($n=8$), FAT: Ex+FR ($n=8$). FAT did not affect the representative digestive and absorptive molecular expression of lipids and proteins. FAT, female athlete triad.

absorption molecules are markedly reduced when the intestinal villus is atrophied[12,19]. However, in the present model, there was no fasting or interruption of stimulation of the intestine, but rather a restriction on the amount of food consumed; therefore, the decrease in these molecules did not occur. On the contrary, interestingly, gene and protein expression of sucrase-isomaltase, a typical carbohydrate-digestive enzyme, were significantly increased only in the FAT group (Fig. 5). Sucrase activity also tends to increase, although not significantly (Fig. 5). Although the results may seem contradictory, as Hampson reported that sucrase activity correlates with villus height in the small intestine[32], several studies have reported that

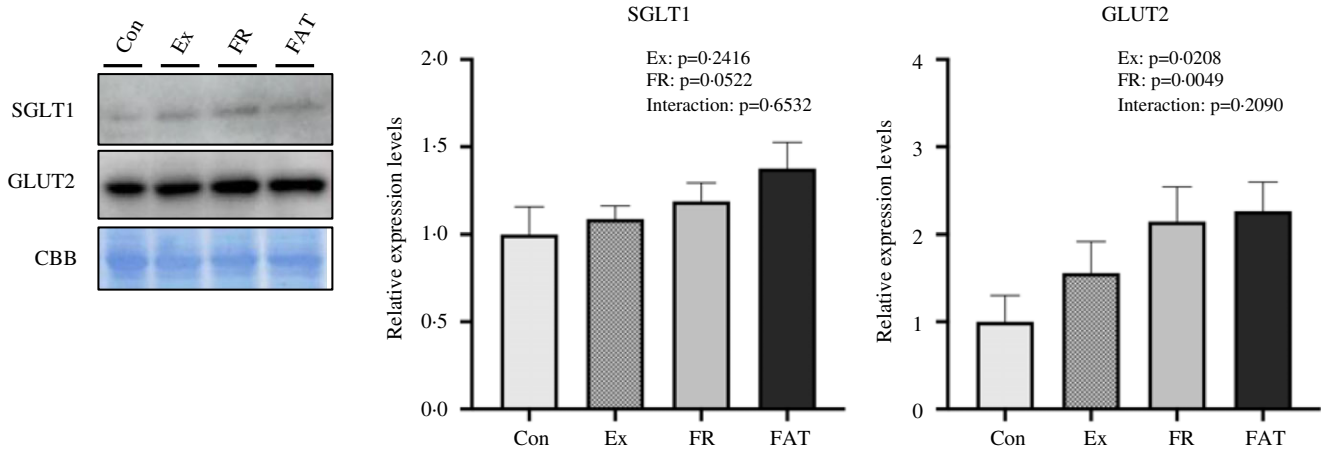


Fig. 4. The effect of continuous food restriction and exercise on the expression levels of monosaccharide transporters. Blotting images show representative images. All data are presented as mean ± SEM. SGLT1: Na^+-dependent glucose transporter 1, GLUT2: Glucose transporter 2, CBB: Coomassie brilliant blue stain. Con: control ($n=7$), Ex: exercise ($n=7$), FR: food restriction ($n=8$), FAT: Ex+FR ($n=8$). There were no significant differences in the protein expression of sodium-glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2), which are typical glucose transporters. FAT, female athlete triad.

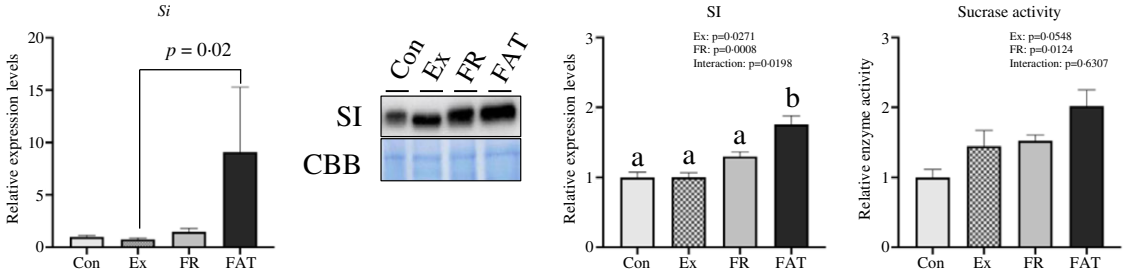
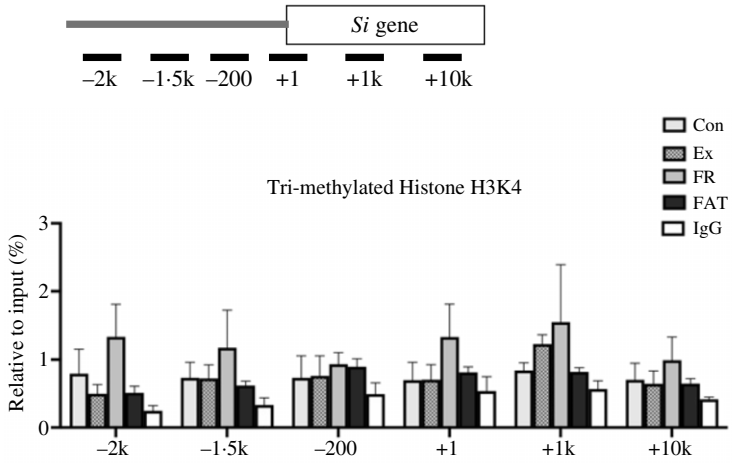


Fig. 5. The effect of continuous food restriction and exercise on the expression levels of sucrase-isomaltase gene (*Si*) and protein (SI) and sucrase enzyme activity. Blotting images show representative images. All data are presented as mean ± SEM. Different letters indicate significant differences. SI: sucrase isomaltase, CBB: Coomassie brilliant blue stain. Con: control ($n=7$), Ex: exercise ($n=7$), FR: food restriction ($n=8$), FAT: Ex+FR ($n=8$). *Si* gene expression was significantly higher in the FAT group than Ex. SI protein expression was significantly higher in the FAT group than in the other three groups. FAT, female athlete triad.

dietary restriction increases disaccharidase activity. Dietary restriction in growing broilers has been reported to result in increased sucrase activity, which also affects subsequent growth rates[33]. In addition, dietary restriction in late gestation sheep has been reported to increase disaccharidase activity[34]. The authors stated that a decrease in small intestinal mass increases disaccharide enzyme activity, suggesting a decrease in energy utilisation by portal-discharging viscera[35], which may result in increased digestive efficiency[33,36]. Together, the results of the previous studies and the present study suggest that dietary restriction and increased energy demand are conserved phenomena that increase sucrase and other disaccharidase enzyme activities in various species, and it is highly likely that a similar phenomenon occurs in FAT in humans. In addition, blood glucose levels tended to be lower in the FAT group than in the other three groups, although the difference was not significant (Table 2). Among the three macronutrients, the body uses carbohydrates and lipids as primary energy substrates. Carbohydrates, which are easily converted into energy, are in particularly high demand as energy substrates in the body during exercise. Therefore, the effect on SI may be a biological response to efficiently digest carbohydrates in order to obtain more glucose. Finally, we focussed on the regulation of SI and analysed the methylation of the *Si* gene. Although the normal expression of SI is important for disaccharide digestion, its overexpression is a risk factor for diabetes and obesity. In fact, SI expression and activity are increased in the small intestine of diabetic patients and animals[37–40]. On the other hand, athletes are concerned about excessive weight gain after retirement[41,42]. We hypothesised that dietary restriction and excessive exercise during playing days may cause methylation in the *Si* gene, which may persist post-retirement, harming the health of the athlete. However, contrary to this hypothesis, there were no significant changes found in the *Si* gene regions in any of the groups (Fig. 6). The only methylation measured in this study was trimethylation of histone H3K4. Other histone modifications such as mono- and demethylation were not identified in this study. Furthermore, methylation and acetylation of the histone region may also be involved in the epigenetic regulation of *Si* gene expression. Indeed, it has been reported that inhibition of histone deacetylases increases *Si* gene and protein expression[43]. In addition to histone modifications, there are other factors that may regulate *Si* gene expression, such as microRNAs (miRNA). Mehraban *et al.* reported that miRNA-26a and miRNA-26b decrease *Si* gene expression and enzyme activity[44]. Therefore, it is possible that the *Si* gene is also



Region(bp)	Ex p-value	FR p-value	Interaction
−2k	0·3429	0·3770	0·4423
−1·5k	0·7906	0·5143	0·8389
−200	0·8965	0·5614	0·9966
+1	0·7493	0·3193	0·6174
+1k	0·1639	0·5510	0·3335
+10k	0·6578	0·4990	0·9576

Fig. 6. The effect of continuous food restriction and exercise on the expression levels of methylated histone H3K4me3 in the *Si* gene. All data are presented as mean ± SEM. Si: sucrase isomaltase. Con: control ($n = 4$), Ex: exercise ($n = 4$), FR: food restriction ($n = 4$), FAT: Ex+FR ($n = 3$). There were no changes in histone H3K4 trimethylation in any region of the *Si* gene. FAT, female athlete triad.

regulated in FAT by histone modifications in other regions and by miRNA regulation, which was not examined in this study. Therefore, future studies targeting such mechanisms would provide more information on the effects of excessive exercise and strict dietary restrictions on the intestinal tract.

This study has several limitations. First, the model in this study was a short-term, 4-week model with more severe dietary restrictions. Since most previous studies have involved interventions of approximately 14 weeks with 70 % dietary restriction, it is necessary to examine whether similar results can be obtained with such a model. Second, only typical digestive enzymes and transporters were examined. In addition to digestion and absorption, the intestine has many other functions (such as immunity and intestinal barrier functions).

Conclusions

To the best of our knowledge, this is the first study to show how FAT affects the small intestine. In this study, the FAT model induced villus atrophy in the small intestine but inversely increased the expression of SI gene and protein. These results may provide insights into the future conditioning of weight-sensitive athletes and the maintenance of health post-retirement.

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K. A., H. S. and K. E. prepared the first draft of the manuscript. K. A., H. S. and N. O. designed the experiments. K. A., K. E., H. S. and K. K. performed the experiments. K. A., K. E. and H. S. analysed the data. T. S. provided an experimental technique. Y. K., K. T. and N. O. supervised all the experiments. All authors have qualified for authorship and have been listed.

The authors declare no conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have influenced the study.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114522003063

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