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ORIGINAL ARTICLE

Effects of Treadmill Exercise on Fundus Ghrelin Expression and Plasma Acylated Ghrelin level in Male Rats

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The gastric peptide ghrelin, an endogenous ligand for growth-hormone secretagogue receptor, in its acylated form induces a positive energy balance, increase in food intake and adiposity. This study aimed at determining the effects of treadmill exercise training on fundus Ghrelin mRNA expression, fundus, and plasma acylated Ghrelin concentration in rats. Materials and Methods: Thirty-six adult Wistar male rats (12-14 weeks old, 200-220g) were randomly divided into the experimental 1 (EX1) (n=16) and experimental 2 (EX2, n=20) groups with further division into control (n=8 and 10) and training (n=8 and 10) groups. Training groups were given exercise on a motor-driven treadmill (28 m/min, 0% grade, 60 min, 5 days/week for 8 weeks). Rats in EX1 were further divided into four groups; fed-control (FEC), fed-trained (FET), fast-control (FAT) and fast-trained (FAT). Twenty-four hours after last training session, the fundus was excised and frozen in liquid nitrogen for extraction of ghrelin mRNA. Fundus and plasma acylated ghrelin, growth hormone (GH), insulin, cortisol, lipids, and glucose were also measured. Results: Ghrelin mRNA expression was significantly (P=0.002) higher in fasted rats and lower in trained-rats, in whom a non significant increase was observed in resting plasma acylated Ghrelin, GH, insulin, liver glycogen and lower free fatty acids concentrations and muscle glycogen. Plasma cortisol, triglycerides (TG), total cholesterol (TC) was remained unchanged. Interestingly, fundus acylated ghrelin was significantly (P=0.031) lower in trained rats. Conclusion: The data obtained showed that treadmill exercise reduced ghrelin expression and its acylated levels in the fundus of trained rats and a higher plasma acylated ghrelin could be released from other source (s).

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Introduction

Ghrelin, a 28-amino acid peptide recently isolated from the human and rat stomach, is also present in human and rat pancreatic alpha-cells[1, 2]. It is also recognized as a novel player in the gut-brain regulation of growth hormone and energy balance[3]. The molecule has been shown to be a growth hormone (GH) secretagogue that stimulates an increase in blood glucose[4,5]. Ghrelin has a potent effect on eating behavior, causing an increase in hunger[2] and plays a key role in the central regulation of feeding[6]. It has been suggested that the stomach is a major source of circulating ghrelin in humans[7]. Interestingly, ghrelin levels are significantly altered during acute and chronic aberration in

nutritional status. Thus ghrelin levels are low in simple obesity but increase after weight loss[8,9]. Ghrelin is up-regulated by fasting, insulin-induced hypoglycemia and leptin administration[10,11]. Plasma Ghrelin response to different kind of physical exercise has been studied by several investigators in human[12,13], horse[14,15], and rat subjects[16,17]. The results are conflicting, several studies documenting no change in plasma ghrelin concentration, while others observed suppressed levels. To date, most investigations have focused on plasma ghrelin response to an acute exercise (short-term, or long-term, resistance or aerobic exercise). Foster-Schubert et al studied the effect of an aerobic exercise for 12 months on 173 sedentary, overweight, postm-enopausal women, and reported that plasma ghrelin increases by 18% in exercisers who lost over 3 kg of weight[13]. Ebal et al[17] studied the effect of 5 weeks of a force-resistance exercise on plasma ghrelin concentration in rats, and suggested that training reduced the rats' body weight by 6.4% and lowered ghrelin levels. Recent evidence suggests that skeletal muscle is an important site of ghrelin action. Ghrelin and growth hormone secretagogue receptor (GHS-R) have been found in skeletal muscle[18] and it has been shown that growth hormone secretagogue can alter both electrical and contractile activity in skeletal muscle[19]. Moreover, GHS receptors have been localized in skeletal muscle[20]. Collectively, these studies suggest that the fundus as well as skeletal muscle is important sites of ghrelin action.

Treadmill running speeds have been used previously to elicit the respective moderate VO_2 max values (%) for Wistar rats[21] and previous data from our lab suggest that training, 25m/min, 60 min/day, 5days/week for 6 weeks affected total ghrelin levels in rat soleus muscle[22]; Hence we utilized a reasonable training time period to produce the expected acylated ghrelin adaptations. However, there is no information about the effects of treadmill exercise (8 weeks) at moderate intensity (28 m/min, 0% grade) on fundus ghrelin expression, fundus and plasma ghrelin concentration in wild type male rats. This study was therefore conducted to determine the effect of treadmill exercise on resting fundus ghrelin expression, fundus plasma ghrelin concentrations and gluco-regulatory hormones.

Materials and Methods

Animal experiments

All experiments involving the animals were conducted according to the policy of the "Iranian Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes," and the protocol was approved by the Ethics Committee of the School of Medical Sciences, Tarbiat Modares University (TMU), Tehran Iran. Thirty-six Wistar male rats (12-14 weeks old) weighing 200-220 grams were used for this study. Animals were obtained from the Pasteur Institute (Tehran, Iran) and maintained in the Central Animal House of the School of Medical Sciences, TMU. The animals were housed five per cage; light was controlled on a 12:12-h light-dark cycle, temperature was 22.0±1.4°C and humidity was 55.6±4.0%. Animals were fed a pellet rodent diet ad libitum and had free access to water. Strewment was changed every 4 days, and the same person handled the rats throughout the study. Animals were randomly assigned into the EX1 (the first experimental, n=16) and EX2 (the second experimental, n=20) groups. All experi-mental animals were also randomly divided into control group, which remained sedentary (Con), while the training group (TR) underwent a moderate running exercise program. The day before the experiment, animals in EX1 group were randomly further divided into 4 groups; fed-control (FEC), fed-trained (FET), fast-control (FAC), and fast-trained (FAT), 4 animals in each. Rats were fasted overnight (at least 8 hours), with free access to water. All Animals in EX2

group were treated similar to the EX1 group, except for further division.

Exercise training protocol

Treadmill training began with familiarization of rats with the apparatus for 4 days by placing them on the motorized driven treadmill (Iranian Model, designed by, Physical Education & Sports Sciences Department of TMU, and Tehran, Iran). Training group was given exercise training for 5 days/week for 8 weeks as described previously[23,24]. In the first week, rats were exercised on the treadmill at a speed of 10 m/min, angle of inclination 0% gradient and the running time was 15 min/day. In the second week the speed was increased to 24 m/min, with 0% gradient and the duration to 45 min/day. In the third week, the speed was increased to 28 m/min, with 0% gradient, while the duration remained at 45 min/day. In the fourth week, the speed remained 28 m/min, with 0% gradient and the duration increased to 60 min/day. In the last 4 weeks the speed and duration remained constant. This condition corresponded to a moderate intensity of about of 65%-70% of maximal oxygen consumption[23]. The animals were not exercised for 24 h prior to sacrifice.

For biopsies and blood sampling, twenty-four hours after the last training session (8th week), rats were anesthetized intra-peritoneally with a mixture of Ketamine TM (30-50 mg/kg body wt, ip) and Xylazine (3-5mg/kg body wt, ip), and the fundus part of stomach, liver, and the entiregastrocnemius muscle were excised, each divided into two pieces and washed in ice-cold saline, and immediately frozen in liquid nitrogen for extraction of ghrelin mRNA, fundus acylated ghrelin concentration, liver and gastrocnemius muscle glycogen contents. All frozen fundus, liver and muscle pieces were stored at -80°C until analyses were performed. Liquid nitrogen homogenate of the tissue (100mg/mL of Phosphate Buffer Saline) were used for biochemical analysis. Blood was collected in EDAT test tubes as anticoagulant and immediately processed for plasma preparation, during 10min centrifugation at 3000rpm. Plasma was aliquted and stored at -80C for future analysis.

Extraction of mRNA and RT-PCR quantization

The quick frozen fundus part of the stomach was powdered in a cold mortar and pestle, and approximately 50 mg was used for the isolation of RNA. Total RNA was extracted by the Guanidinium Thiocyanate method[25] and mRNA purified using mRNA Isolation Kit (Roche, Germany) according to manufacturer instructions. One microgram of mRNA was used to synthesis first strand cDNA in a 20ml volume by using oligo (dT) primer in the first-strand synthesis kit (Fermentase, Germany). Relative expression level of Ghrelin mRNA in the muscle was determined using a semi quantitative PCR method. The following primers were used to amplify rat Ghrelin and Beta-actin (as an internal control) cDNA: Ghrelin-Forward: 5′-TTGAGCCCAGAGCACCAGAAA, Ghrelin -Reverse: 5′-ACT TGT TAG CTG GCG CCT CTT TG-3'. Beta-actin-Forward: 5′-TCC TGT GGC ATC CAT GAA ACT-3′; B-actin-Reverse: 5′-ATC GTG CAC CGC AAA TGC TTC-3′. Ghrelin cDNA was amplified giving a 270-bp product. PCR was formed for 35 cycle of denaturation 94ºC for 30s, annealing of 58ºC for 30s and extention at 72ºC for 50s. Reactions were set up using a 2 fold serial dilution of template cDNA to assess the best dilution of template in PCR. Template cDNA was standardized by amplification of a 315-bp internal control of B-actin, a house keeping gene, a protein known to be expressed in the tissues. All the reactions were repeated a minimum of three times to ensure repeatability. All PCR products were electrophoresed on an agarose gel and bands visualized by ethidium bromide staining and quantitated by computer integrated densiometry (Kodak, CT). Levels of mRNA were expressed as a ratio of signal intensity for the B-actin gene.

Biochemical analyses

The samples were analyzed for plasma and fundus acylated ghrelin, glucose, GH, insulin, cortisol, triglycerides (TG) total cholesterol (TC), and free fatty acids (FFA). Plasma high density lipoprotein cholesterol (HDL) was determined by the direct immuno method (HDL-C Immuno FS, Pars Azmoun, Tehran, Iran), the intra-assay coefficient of variation and sensitivity of the method were 1.2% and 0.03mmol/L respectively. Plasma total TG was determined using the enzymatic (GPO, glycerol-3-phosphate oxidase) colorimetric method (Pars Azmoun, Tehran, Iran). The intra-assay coefficient of variation and sensitivity of the method were 2.2% and 1 mg/dL respectively. Plasma total cholesterol (TC) was determined by the enzymatic (CHOD-PAP, cholesterol oxidase-amino antipyrine) colorimetric method (Pars Azmoun, Tehran, Iran), the intra-assay coefficient of variation and sensitivity of the method being 1.9% and 0.08 mmol/L, respectively. Liver and gastrocnemius muscle glycogen were determined by a commercial kit (Glycogen Colorimetric kit, Nanjing, China)

Statistics

All results are expressed as mean ±SEM. All variables were compared by unpaired t-test. Correlation was calculated using the Pearson Product Moment correlation. All statistical analyses were performed by using SPSS (Version 13). All p values <0.05 were considered significant.

Results

Fundus ghrelin gene expression

Semi-quantitative RT-PCT technique showed in Fig.1 including the ghrelin expression in fed-control, fed-trained, fast-control, and fast-trained rats.Overall, the overnight fasted rats showed a higher and significant (P=0.03) fundus ghrelin expression (Fig.1), whereas the trained group had lower ghrelin expression when compared to the control group (Fig. 2).

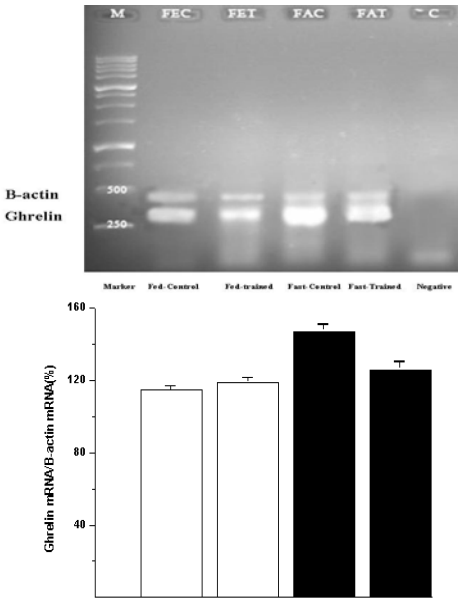

Fig.1. Semi-quantitative reverse-transcription PCR (RT-PCR) of ghrelin mRNA in fundus in fed-control (FEC), fed-trained (FET), fast-control (FAC), and fast-trained (FAT) male rats, 4 animals per group

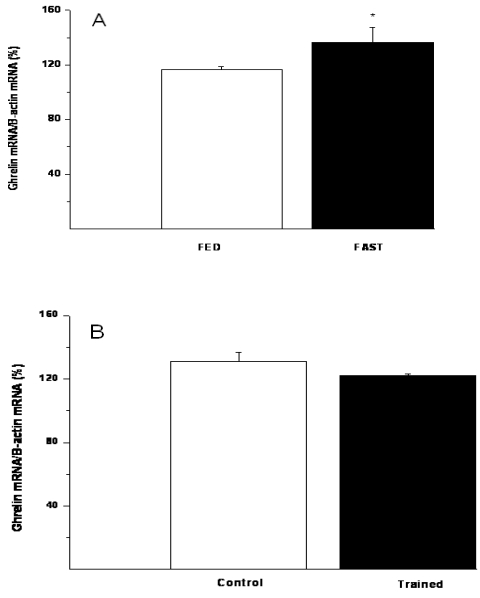

Fig.2. Fundus ghrelin mRNA expression in fed, fast (A), control, and trained wild type (B) male rats, N=10 animals of each. Data is expressed as mean ± SEM. * fast vs fed, P=0.002.

Table 1. Anthropometry, plasma glucose, hormones, and lipid profiles in control and trained wild type male rats

Variables	Control Group n=10	Trained Group n= 10
Weight (g)	345.00 ± 12.63	321.5± 8.1
Height (cm)	23.70 ± 0.25	23.43 ± 0.45
BMI (kg/m^2)	6.12 ± 0.12	5.84 ± 0.10
Cortisol (µg/L)	2.92 ± 0.45	2.51 ± 0.59
TG (mg/dL)	131.8 ± 5.5	131.0 ± 4.7
TC (mg/dL)	121.8 ±6.7	118.25±7.30
FFA (µg/L)	567 ± 65	469 ±31.5
Glucose (mg/dL)	137.13 ± 10.40	133.63 ±8.9
Gastrocnemius Glycogen (mg/g)	43.02 ± 4.10	33.77 ± 3.01
Liver Glycogen (mg/g)	79.42 ± 10.55	92.85 ± 8.94

Resting plasma ghrelin was non-significantly higher in the trained group, when compared to controls (18.55±3.92 pg/m1 vs. 12.51±1.80pg/mL) (Fig.3). In contrast to plasma, resting fundus ghrelin concentration was significantly (P=0.039) lower in trained rats, when compared to the untrained group (9.24±2.47pg/mL vs. 20.67±4.20pg/mL) (Fig.3).

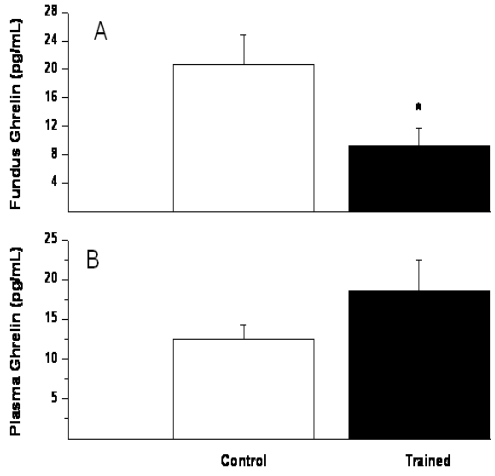

Fig.3. Fundus (A) and plasma (B) acylated ghrelin concentrations in control and trained wild type male rats, n=10 animals each. Data is expressed as Mean±SEM, *trained vs. control, P=0.03.

Plasma glucose, cortisol, GH, and insulin concentrations

Changes in resting plasma glucose, and cortisol were not significant (Table.1). Resting plasma GH and insulin concentrations were non-significantly higher (approx 1.33 and 2.408 fold, respectively) in trained rats when compared to the control group (P=0.41, P=0.214, respectively) (Fig. 4).

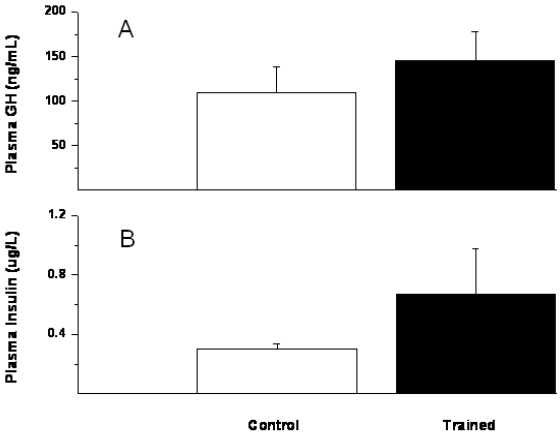

Fig.4. Plasma GH (A) and insulin (B) concentrations in control and trained wild type male rats

Plasma triglyceride (TG), total Cholesterol (TC), and free fatty acid (FFA)

Plasma TG remained unchanged. Total cholesterol concentration was non-significantly lower in trained rats. A considerable reduction of plasma FFA did not reach significant level in trained rats (Table 1).

Liver and muscle glycogen concentrations

Resting liver and whole gastrocnemius muscle glycogen concentrations were non-significantly higher (92.85±8.94 vs. 79.42±10.55mg/g wet tissue) and lower (33.77±3.01 vs. 43.02±4.15mg/g wet tissue) respectively, in trained rats when compared with controls (Table 1). There were no significant correlations between plasma

acylated ghrelin and variables measured (Table 2).

Table 2. Baseline Pearson correlation coefficients of acylated ghrelin levels with body weight, BMI, fundus acylated ghrelin, lipid profile, hormones, liver and muscle glycogen concentrations in wild type male rats.

Variables	Correlation with plasma ghrelin	P values
Weight (g)	- 0.056	0.836
MBI (kg/m2)	0.129	0.633
Fundus acylated ghrelin (pg/mL)	- 0.070	0.819
Glucose (mg/dL)	0.483	0.068
TG (mg/dL)	- 0.075	0.781
TC (mg/dL)	- 0.023	0.934
FFA (µg/L)	- 0.107	0.703
GH (ng/mL)	- 0.256	0.338
Insulin (µg/L)	- 0.108	0.690
Cortisol (µg/mL)	0.151	0.576
Gastrocnemius glycogen (mg/g)	-0.061	0.842
Liver glycogen (mg/g)	0.493	0.134

Discussion

The major findings of the present study were a significantly higher ghrelin expression by fasting, a lower ghrelin expression and fundus acylated ghrelin by treadmill exercise. Correlations between plasma ghrelin and plasma glucose concentration and between fundus ghrelin and TG were other important findings. However a non-significant and higher plasma acylated gGhrelin, GH and Insulin were obtained. Fasting for different durations (18, 24, 48, 72 hours, and 7days) is also known to be associated with increased plasma ghrelin concentrations and expression of ghrelin in the gastrointestinal tract, mainly gastric and particularly in the fundus[26-28]. According to Ghelardoni et al.[29], fasting did not result in a major changes in stomach ghrelin mRNA expression, but ghrelin protein concentration in tissue was significantly modified in fasted rats; they suggested that while ghrelin concentrations were increased in most tissues, they were decreased in the stomach, accompanied with an increase in plasma ghrelin concentration in fasted rats. Data on the effect of treadmill exercise on ghrelin mRNA expression and acylated ghrelin levels in wild rats' fundus are lacking. The present study, for the first time, showed that, resting ghrelin mRNA expression and its levels in rat fundus decrease, following the 8 weeks of treadmill exercise training. The mechanism(s) by which treadmill exercise reduces fundus ghrelin concentrations is poorly understood. However, it is believed that the synthesis and release of ghrelin from the gastrointestinal tract, particularly the stomach as a main source, and the splanchenic bed, as a minor source of circulating ghrelin, could be regulated by nutrients and hormones, such as insulin, GH, glucagon, and glucagon-like peptide[30-37]. Exercise is a known stimulant of growth hormone secretion and the magnitude of elevation has been found to be related to both the duration and intensity of the exercise[38,39]. Increases in growth hormone levels after endurance exercise have been well documented[40-43]. In the present study trained rats had higher and nonsignificant resting plasma GH (1.36 fold) and insulin (2.24 fold) concentrations. Actually, we do not know that how much change of the endogenous GH and insulin are required to inhibit ghrelin secretion from stomach. In this study we did not measure glucagon-like peptide-1 (GLP-1), but, it is believed that ghrelin secretion for stomach could be inhibited by GLP-1 which is released from intestinal L-cell. O'Connor et al[38]. suggested that running on a treadmill for 2 h at 60% VO2 max significantly increased gastrin, and glucagon-like peptide-1 (GLP-1). Martines et al[39]. has reported that 60 minutes of cycling at intensity of 64-68.5% heart rate maximum significantly increased mean PYY, GLP-1, and pancreatic polypeptide (PP) during exercise and this increase was maintained during the post-exercise period for GLP-1 and PP. In addition to hormonal regulation of ghrelin, it is believed that cholinergic

(acetylcholine) and sympathetic system plays an important role in ghrelin secretion from gastrointestinal tract[40-42]. Mundinger et al.[43] observed that the electrical stimulation of postganglionic sympathetic axon projecting from the celiac ganglion and using a sympathomimic drug (Tyramine, TYR) raised portal ghrelin concentrations in rats. On the other hand, gastric/intestinal electrical stimuli for systemic release of hormone, gut peptides secretion, plasma glucose, and food intake by previous studies[44-46]. In study by Xing et al.[45] long-pulse (300 msec) electrical stimulation showed that the total AUCs of plasma ghrelin was not significantly affected but after 2hours of an electrical stimulation on gastric (GES) (long pulse-with pulse trains) and duodenal electrical stimulation (DES) (with pulse trains) significantly and dramatically decreased fundus ghrelin. An endurance-exercise induced adaptations on different body systems and organs; particularly on gastrointestinal has been studied by several investigators [45-48]. A lower ghrelin mRNA expression and fundus acylated ghrelin and a higher plasma acylated ghrelin levels could be attributed to metabolic changes (liver and muscle glycogen and ATP depletion are less in endurance trained species) and might be due to an incomplete energy source recovery in trained and overnight fast rats. A higher plasma acylated ghrelin in the present study might be attributed to a liver and skeletal muscle glycogen and ATP deficiency and an incomplete recovery of energy sources after the last exercise session. In the present experiment conditions resting liver and whole gastrocnemius muscle glycogen conce-ntrations were nonsignificantly higher and lower in trained rats, respectively. Although, a limitation of the study with regard to this finding is that total ghrelin concentrations were not measured, but an increase in fundus acylated ghrelin catabolism and conversion to other forms of ghrelin and also a reduction in stomach and fundus ghrelin-expressing cell dense cannot be excluded. To our knowledge, the present study is the first to examine the resting plasma acylated ghrelin concentrations after 8 weeks of treadmill exercise training in rats. However several previous studies the response of plasma total ghrelin to exercise training program in human[13] and horse[14-15] have investigated. In study by Tschop et al.[26] in rats were subjected to an acute treadmill running (22m/min, 10% slope) resulted in a significant increase in plasma total ghrelin level which was accompanied with a significant reduction in liver and gastrocnemius (white and red) muscle glycogen contents. In study by Ebal et al.[17] a 5 weeks of force-resistance exercise resulted in a significant reduction in plasma total Ghrelin concentrations in fed rats. The discrepancies between our results with previous reported results could be explained by subjects (human vs. rats), duration of exercise training program (8 weeks vs. 5 weeks), mode of exercise training (treadmill running vs. force-resistance exercise), and nutritional status (overnight fast vs. fed condition). It seems that higher resting plasma ghrelin in trained rats might be due to an incomplete recovery of energy sources which become depleted during the last session of treadmill exercise which was combined with an overnight fast state in rats[49-55]. A lower muscle glycogen as we observed in the present study might be considered as a stimulus for this elevated plasma acylated in trained rats. Despite treadmill-exercise induced reduction in fundus acylated ghrelin, this elevated plasma acylated ghrelin level might be due to an increase in ghrelin secretion from extra-gastric tissue such as splanchenic beds[55,56] and peripheral blood lymphocytes (our unpublished data). This is the first report demonstrating that resting fundus ghrelin expression and its acylated ghrelin lower in trained rats. The present study also for the first time provides information about resting plasma acylated ghrelin in wild type male rats.

In summary, the data indicate that a treadmill-exercise induced decrease in fundus ghrelin expression was accompanied with a reduction in fundus acylated ghrelin, but not in plasma acylated ghrelin. The results also indicate that an overnight fasting-induced deficiency in muscle and liver energy status might be considered as a factor in ghrelin regulation. It seems that the effect of treadmill exercise training on ghrelin was somewhat masked by overnight fast period. A higher resting plasma acylated ghrelin level in trained rats in partly confirms the secretion of ghrelin from the other sources. It can also be concluded that treadmill-exercise training was able to modulate this orexigenic peptide.

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