

Ghrelin concentration in half-blood jumping horses during moderate and intense training exercise

Hanna Bis-Wencel, Krzysztof Lutnicki¹, Agnieszka Zofia Rowicka,
 Monika Bryl, Bożena Nowakowicz-Dębek

Department of Animal and Environment Hygiene,
¹Department and Clinic of Animal Internal Diseases,
 University of Life Sciences in Lublin, 20-033 Lublin
 hanna.biswencel@up.lublin.pl

Received: May 16, 2013

Accepted: September 16, 2013

Abstract

The aim of the study was to assess the impact of varying intensity of exercise on ghrelin levels in half-blood jumping horses during training season. Blood samples were collected in the period of intense and moderate exercise. Serum concentrations of active and total ghrelin were measured using commercial RIA kits. Exercise did not cause the disruption in energy homeostasis, which was confirmed by the conducted tests. Total concentration of ghrelin measured during the increased effort intensity elevated in stallions (from 1031.45 to 1732.19 pg/mL at $P \leq 0.05$). The opposite situation occurred in the case of active ghrelin, which concentration did not increase significantly in mares (from 26.21 to 28.46 pg/mL), whereas an insignificant decrease was noted in stallions (from 24.19 to 22.54 pg/mL). The only significant difference between stallions and mares was noted in the 2nd sampling ($P = 0.015$). The research proved that the fluctuations of both studied parameters did not reflect a negative impact of the increased effort.

Key words: horses, ghrelin, exercise.

Introduction

Intensive training and effort to which horses are subjected, can be the cause of failure in energy balance maintenance. This can lead to health problems, because prolonged negative energy balance is related to a decreased physical activity, growth rate, and milk production (21). Ghrelin is an orexigenic hormone produced primarily in the X/A-like cells of the stomach (4). It increases due to meal anticipation or initiation in both humans and animals (2). On the other hand, ghrelin is the only protein known in animals to be modified by O-acylation with octanoate, the eight-carbon fatty acid. Although octanoylation is required for the endocrine actions of ghrelin, the enzyme that catalyses the modification has not been identified yet (19). The discovery of ghrelin was reported in 1999 by Kojima *et al.* (19), who were searching for a protein that stimulates the secretion of growth hormone in the pituitary gland. The ligand was purified from rat stomach, and it was shown to stimulate the release of growth hormone from cultured pituitary cells. Kojima *et al.* (19) have determined that the 28 amino acid

ghrelin is derived proteolytically from a precursor of 117 amino acids. The analysis by mass spectroscopy revealed that serine-3 of ghrelin is modified by O-acylation with an octanoyl group, which is required for growth hormone releasing activity. Serine-3 is conserved in mammals, birds, and fish. In ghrelin of the bullfrog, serine-3 is replaced by threonine, but this residue is also octanoylated (26). The peripheral ghrelin concentration is negatively correlated with body fat in humans (13, 24) and horses (7, 8). In recent years, the research on horses has been focused on the relation between energy homeostasis and exercise. The energy intake and expenditure is a very important factor in terms of horses conducted to different, various exercises. Inadequate energy balance may affect the best equine athletes, causing failure in training, due to high energy requirements (6). It was also shown that peripheral hormonal signals such as ghrelin, play a crucial role in mediating energy homeostasis, because of their effect on feed intake, glucose and insulin regulation, and fatty acid metabolism (5, 9). Ghrelin also stimulates growth hormone release from the pituitary gland. However, more data on the physiology

of this energy balance mediator is necessary, since underweight or overweight can be crucial for health and performance of horses (17, 21). The short, exhaustive exercise demonstrated no changes in ghrelin concentration during, or shortly post exercise, but the data concerning ghrelin fluctuations during the planned and different effort is not complete. Both moderated and intense exercises can interfere with the ghrelin total and ghrelin active concentrations and the maintenance of energy balance (7). Mares, stallions, and geldings often take part in the same races or competitions regardless of sex (18) and the concentrations of the total and active forms of ghrelin may serve as indicators of energy sufficiency, providing valuable information on the current state of energy balance (9). In the present study acylated form of ghrelin, known as “active ghrelin,” was measured because it is considered as the biologically active form (19). It was assumed that active ghrelin, adiponectin, leptin, glucose, and insulin would demonstrate a diurnal variation during the control of energy homeostasis in horses (6). The aim of this study was to assess the impact of varying intensity exercise on the ghrelin levels in half-blood jumping horses during training season.

Material and Methods

Research was conducted on 18 clinically healthy half-blood horses (b.w. = 500 ± 70 kg), with high training advancement, competing in show jumping discipline. The experimental group consisted of 10 stallions (8 ± 2 years of age) and eight mares (10 ± 2 years of age). The study was conducted in the spring-winter season. The horses were kept in separate stalls in the stables and had a permanent access to tap water. Average ration per day per one horse was 3.5 kg of hay twice a day, 5 kg of oats (in two portions), and 60 g of vitamins B and E (Hipp Vital). During intensive exercise, horses were given extra feed in the form of muesli (Dynamix's Besterly), 1 kg twice a day. The daily training programme consisted of 1 h on carousel, 10 min walk, 10 min of relaxed trot, 5 min of working trot, and 7 min of galloping. The training sessions were enhanced by an additional 1 h of lunging and 1 h of riding under saddle during the intense exercise period. Blood samples were collected in the period of moderate (1st collection) and in the period of intense exercise (2nd collection). Blood was taken from the external jugular vein to dry tubes, in a closed system, always at the same time, before feeding, watering, and any kind of effort. All blood samples were immediately centrifuged at $3000 \times g$ for 10 min, aliquoted, and frozen at -80°C . Serum active ghrelin and total ghrelin concentrations were measured using commercial RIA kits (Millipore, USA). The intra assay CV for active ghrelin and total ghrelin kits were $<10\%$. The active ghrelin kit comprised ^{125}I -labeled ghrelin with a specific activity of

302 $\mu\text{Ci}/\mu\text{g}$, the 0.025% Triton-X 100 with normal guinea pig IgG as a carrier, and goat anti-guinea pig IgG antibodies, 3% PEG and 0.05% TritonX-100 in 0.005 M in the precipitating reagent. The total ghrelin kit consisted of ^{125}I -labeled ghrelin with a specific activity of 302 $\mu\text{Ci}/\mu\text{g}$, the 0.025% Triton-X 100 with normal rabbit pig IgG as a carrier, and goat anti-rabbit pig IgG antibodies, 3% PEG and 0.05% TritonX-100 in 0.005M in the precipitating reagent. Samples were examined in duplicate and counted in a γ -counter (Agroecological Laboratory in Lublin, Poland). The information from the manufacturer indicated a specificity of 100% for human, rat, and canine active ghrelin, and $<0.1\%$ for des-octanylghrelin in the case of active ghrelin, and a specificity of 100% for human, rat, canine, and des-octanylghrelin for the total ghrelin. The accordance of the results of the active ghrelin assay kit and total ghrelin assay kit was established using a serial dilution of horse plasma and the ghrelin standard from the particular assay kit, as was previously described for active and total ghrelin validation (6, 17). Results were expressed as human equivalents of immunoreactive ghrelin.

The obtained results were expressed as arithmetic means ($\bar{x}$) and standard deviations (SD), and subjected to statistical analysis. The significance of differences between mean values were calculated using Student's *t*-test for dependent variables, Microsoft Office Excel 2007. The statistical significance limit was considered as $P \leq 0.05$.

Results

Mean values of total and active ghrelin are presented in Table 1. The mean concentrations of total ghrelin varied between the collections (1091.86 pg/mL in the 1st and 1442.04 pg/mL in the 2nd collection) and these differences were statistically significant ($P \leq 0.05$). In stallions, an increase was noted from 1031.45 to 1732.19 pg/mL, which was statistically significant ($P \leq 0.05$); whereas in mares, the downward trend was observed (from 1167.36 to 1079.33 pg/mL), although difference was not statistically significant. Mean concentrations of active ghrelin in all horses maintained at a balanced level (25.08 pg/mL in the 1st and 25.17 pg/mL in the 2nd collection). The concentration had a downward tendency during the collections in stallions from 24.19 pg/mL to 22.54 pg/mL, however, the differences were not statistically significant. On the other hand, the upward trend was noted in mares from 26.21 pg/mL to 28.46 pg/mL but no significant differences were observed. The only significant difference between stallions and mares was noted in the 2nd sampling ($P = 0.015$). The fluctuations of both parameters did not reflect the negative impact of the increased effort.

Table 1. The values of serum concentrations of total and active ghrelin in training horses ($\bar{x} \pm SD$)

Parameters	Collection					
	1 st			2 nd		
	♂	♀	Total	♂	♀	Total
Total ghrelin (pg/mL)	1031.45 ^a ±151.11	1167.36 ±387.29	1091.86 ^c ±280.49	1732.19 ^b ±852.08	1079.33 ±606.46	1442.04 ^d ±804.52
Active ghrelin (pg/mL)	24.19 ±5.58	26.21±8.81	25.08±7.04	22.54 ^a ±4.20	28.46 ^b ±4.35	25.17±5.13

a, b, c, d - mean values differ statistically at $P \leq 0.05$

Discussion

The discovery of the endogenous GHS ligand, ghrelin, has led to new insights into the existence of a hypothalamic-pituitary-gastric axis that provides endocrine control of homeostasis, with growing hormone secretion and gastrointestinal motility. Ghrelin stimulates GH secretion, appetite, and feed intake through the influence on GHR-R_{1a} in the pituitary and hypothalamus (1). A failure to maintain energy balance can lead to health problems and suboptimal performance in horses. Metabolic compounds such as ghrelin, adiponectin, and leptin have been shown to play a role in energy balance, either through their effects on feed intake, fatty acid metabolism, as well as glucose and insulin regulation (5). The concentration of total ghrelin at rest was higher in mares than in stallions in studies by Kędzierski and Kowalik (17, 18), and was equal to 1310 and 1280 pg/mL, respectively. This was confirmed in the present studies. They also demonstrated that concentration of total ghrelin increased significantly ($P \leq 0.05$) in response to continuous exercise as compared to other values, while Kędzierski and Kapica (16) indicated that medium-intensive type of exercise, such as trot, interval, or continuous, slightly affected plasma level of leptin in horses, however significantly affected plasma ghrelin concentration in young Standardbred trotters. The intense exercise significantly increased the plasma concentration of ghrelin measured just after exercise (16). In contrast Gordon *et al.* (9, 10) observed no changes in plasma ghrelin concentration during the exercise test. On the other hand, in humans, exercise decreases or has no influence on serum ghrelin concentrations (13, 25). The study by Leidy *et al.* (20), exploring a relationship between ghrelin and exercise in women reported an increase in ghrelin concentrations and weight loss during the exercise (20). Unfortunately, it turned out that the change was attributed to weight loss or alterations in body composition, because ghrelin did not increase during the exercise, in weight stable control group (8). In contrast to exercise, short-term energy surplus, when dietary energy intake exceeds energy expenditure, has the opposite effect on hormones that regulate energy balance. Three days of energy surplus had no impact on fasting ghrelin concentrations in healthy males and females; yet diet-induced or exercise-induced energy deficit increased ghrelin concentrations to restore

energy balance (11). On the other hand, the study by Dall *et al.* (3) showed no increase in plasma ghrelin concentration during or after 45 min of running exercise at anaerobic threshold in healthy males. Likewise, running at 50%, 70%, 90%, and 100% of maximal oxygen consumption (VO₂max) resulted in no increase in ghrelin concentration in young healthy adults (22). Plasma ghrelin concentration did not increase significantly under exercise conditions, although the increase after the first 30 min of post-exercise almost achieved a significant level ($P = 0.051$). In the study by Jürimäe *et al.* (14) the values obtained in elite male rowers before the exercise were 791 pg/mL below the anaerobic threshold (-AT) and 802 pg/mL above the anaerobic threshold (+AT). The authors found the increasing post exercise tendency of ghrelin 794 pg/mL (-AT) and 808 pg/mL (+AT) and 821 pg/mL (-AT) and 855 pg/mL (+AT), 30 min after effort (14). In the present study on horses, the concentration of total ghrelin increased in stallions after long exercise (from 1031.45 to 1732.19 pg/mL). The results obtained in stallions were statistically significant ($P \leq 0.05$). It seems that high intensity exercise is associated with alterations of leptin and ghrelin contents (7). It is also said that ghrelin may integrate the energy balance by decreasing the lipid oxidation in the liver. Therefore, the increase in serum concentration of total ghrelin in stallions may be due to the economisation of lipids usage in muscles (17). Moreover, the very recent research by Kowalik and Kędzierski (18) confirmed the relationship between the concentration of ghrelin and training intensity. The authors observed that the intensive exercise decreased the serum concentration of total ghrelin. The differences in obtained results among the authors have not been completely explained, however. It might be due to various exercise intensity, to which horses were subjected during the particular research. The design of the equine digestive system might also play a major role in ghrelin concentration (7), as it was demonstrated in immunohistochemical studies, which revealed that ghrelin-immunostained cells were abundant from the neck to the basis of the oxyntic glands of cow, sheep, pig, and horse stomachs. These cells in the stomachs of domestic animals and humans are said to be the producers of biologically active ghrelin (13). Ghrelin is biologically active only in its acylated, active form (19) and may serve as an indicator of energy sufficiency, providing information on the current state of energy

balance. The observations of Gordon *et al.* (6) marked that there was no change in plasma active ghrelin concentration during exercise, what was in agreement with several studies conducted on humans (3, 15, 16, 23). The research exerting various short-term exercises loading, ranging from 10 to 45 min in length, at intensities ranging from the lactate threshold to relative intensities between 50% and 80% VO₂ maximum, all failed to elicit a change in plasma ghrelin concentration during and shortly post exercise (3, 10, 12, 16, 22). Because acute submaximal and maximal physical exercise induce various changes in the hormones of the hypothalamic-pituitary-adrenocortical axis, plasma ghrelin concentration is reduced in obese individuals (13, 24) and substantially elevated in anorexic subjects (10). Other long-term exercise intervention studies have demonstrated that ghrelin levels increase in response to exercise-induced weight loss acting *via* negative feedback loop, which regulates body weight. Acute exercise may induce acute negative energy balance. To date, the published acute short-term submaximal (3, 16) and rigorous (22) exercise studies, investigating ghrelin response, suggest that exercise itself does not alter ghrelin concentration in adults (14). On the other hand, recent studies have shown that strenuous physical cycling exercise for 60 min at 50% of VO₂ max in healthy subjects results in a linear time-related decline in fasted levels of active ghrelin, while the change of total ghrelin plasma levels does not reach significance as measured at various time points during the 60 min exercise period (23). In the study, the concentration of active ghrelin between the 1st and 2nd collection ranged from 25.08 to 25.17 pg/mL. The results are in agreement with the studies of Gordon *et al.* (7) on Standardbred mares, where the active ghrelin concentration increased during exercise ($P \leq 0.05$), as was confirmed in the present study (26.21 to 28.46 pg/mL). Furthermore, a significant difference between stallions and mares in the period of intensive exercise ($P = 0.015$) was also noted. This might have been due to different physiological response and adaptation to effort. In many horses, feed intake decreases after the exercise, and that might be the reason of the higher ghrelin concentrations, resulting from the negative energy balance in racehorses (9, 10). Few studies have shown the ghrelin influence on muscle accretion and long-term growth. Ectopic expressions of ghrelin and its variant were observed 30 d after intramuscular injection, and GH serum concentrations were greater in the injected rats compared with controls. Muscle may have the potential to perform post-translational acylation for ghrelin, and a short ghrelin variant, as described, may have biological effects similar to wild-type ghrelin (1). It is obvious and confirmed in this study, that exercise and training play a role in differences in total and active ghrelin levels between stallions and mares.

There are few studies in which the relation between long-term exercise and ghrelin fluctuations

was investigated (20) thus the problem requires further investigations.

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