Effects of combined training on total ghrelin and tumor necrosis factor-α in obese middle-aged men

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Abstract — Aims: The aim of the present study was to investigate the effects of combined training (CT) on total ghrelin and tumor necrosis factor-α (TNF-α) levels in obese middle-aged individuals. Methods: Twenty two obese middle-aged men (49.32 ± 5.74 years; Body mass index: 30.88 ± 1.64 kg/m²) were randomly assigned to a combined training group (CTG, n = 12) or a control group (CG, n = 10). The CT consisted of aerobic (50–85% of VO₂peak) and resistance (6-10 RM) training performed three times per week, 60 min per session for 24 weeks. The anthropometric measurements, cardiorespiratory test (VO₂peak), maximal strength assessment (1RM) and plasma concentrations of total ghrelin and TNF-α were determined before (Pre) and after 24 weeks (Post) of the experimental period. Results: Decreases were found in body fat percentage (Δ% -19.8) and waist circumference (Δ% -2.8) for CTG at the Post moment as compared to the Pre moment. In addition, the CTG demonstrated increases for VO₂peak (Δ% 13.4) and for 1-RM of bench press (Δ% 78.1), leg press (Δ% 22.3) and arm curl (Δ% 19.3) at the Post moment as compared to the Pre moment. However, total ghrelin levels remained unchanged for CTG and CG after the experimental period, while TNF-α levels increased for CG (p ≤ 0.05). Conclusion: the CT protocol performed was not effective in repairing total ghrelin levels and was not correlated with changes in the TNF-α; however, the exercise training was able to improve body composition and functional capabilities and contained the worsening of systemic inflammation associated to obesity.

Keywords: Obesity; Exercise training; Ghrelin; Tumor necrosis factor-α; Inflammation.

Introduction

Obesity has become a global pandemic1. The World Health Organization2 estimates that 1.9 billion adults are overweight and, of these, over 600 million are obese worldwide. In the case of middle-aged individuals, there is an increase in body fat, especially in visceral adipose tissue, together with decreases in metabolic functions3.

Furthermore, obesity is associated with chronic systemic low-grade inflammation marked by increased levels of inflammatory markers such as interleukin-6 (IL-6), C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α)4. This condition is a common factor in pathogenesis of non-communicable chronic diseases4. In addition, ghrelin, a peptide that exerts potent anti-inflammatory effects on macrophages through negative regulation of the production of the nuclear factor - κβ (NF-κβ)5-7, which is found to be reduced in obese subjects, leading to speculation that it could be one of the possible mechanisms underlying the chronic systemic inflammation associated with obesity8-11. Additionally, ghrelin performs other functions such as the stimulation of growth hormone (GH) secretion12,13, effects on food intake14 and energy balance long-term homeostasis14,13,15.

It is known that regular exercise can promote anti-inflammatory effects in the treatment of chronic systemic low-grade inflammation associated with obesity through different pathways, such as reduction in visceral adipose tissue16, increased blood flow in visceral adipose tissue and decreases in vasoconstriction factors17, increased IL-6 release resulting from muscle contraction18,19, and positive influences on the immune system20,21. Moreover, the return of ghrelin levels to the usual/customary physiological range has been suggested as a mechanism by which exercise training exerts anti-inflammatory effects22,23. The generation of a negative energy balance through training that leads to weight loss and reduction in body fat seems to be involved with the alteration in ghrelin levels5,11. However, the effects of different types of exercise training prescription on ghrelin levels are still controversial and poorly understood. Studies aimed at evaluating the responses of ghrelin to aerobic training (AT) found increases in severely obese adolescents11 and normal-weight young women3 after the experimental period; however, no changes in ghrelin levels were found in elderly9 or middle-aged individuals13 with metabolic syndrome after a period of AT. On the other hand, studies investigating resistance training (RT) did not find significant changes in ghrelin levels in elderly participants with metabolic syndrome13 or overweight/obese24 individuals after the RT protocols.

Moreover, few studies on ghrelin levels have investigated exercise programs that combine both AT and RT [known as combined training (CT)]; CT is recommended by standard positions and guidelines for maintaining and/or improving health condition25, as it can promote the same cardiorespiratory and strength benefits as isolated AT or RT protocols26, as well as, an anti-inflammatory effect on chronic low-grade inflammation associated with obesity21. Recently, Markofski et al.27 observed an increase (40%) in ghrelin levels after 12 weeks of CT in elderly individuals. On the other hand, Kadoglu et al.28 observed no changes in ghrelin levels in obese subjects with type 2 diabetes after 24 weeks of CT. These discrepancies could be related to factors such as age, weight loss and obesity baseline. Similarly, to our knowledge, no previous study has investigated the effects
of CT on ghrelin levels in obese middle-aged men with absence of diseases.

Thus, the purpose of this study was to investigate the effects of 24 weeks of CT on ghrelin levels in obese middle-aged men and its relation to the anti-inflammatory effects of exercise training. We hypothesized that CT would increase ghrelin levels and might influence a reduction in TNF-α levels in obese middle-aged individuals, since ghrelin exerts an anti-inflammatory effect by negative modulation on the production of pro-inflammatory cytokines22.

Methods

Subjects

This study is a secondary analysis of the bigger project on CT in obese middle-aged individuals, whose primary results were published in Brunelli et al.21. The complete trial (ACTRN12615001000594) evaluated the effects of exercise training protocols on physical fitness and metabolic/inflammatory markers in middle-aged overweight/obese men.

Inclusion and exclusion criteria were as follows. Inclusion: the subjects should be middle-aged males who had not participated in regular exercise programs for the previous 12 months according to the Baecke Habitual Physical Activity Questionnaire31 and were classified as obese [(Body Mass Index (BMI) between 30 - 35 kg/m²]. Exclusion: the subjects should be free of coronary artery disease, severe hypertension, diabetes mellitus, chronic obstructive pulmonary disease, limited osteoarticular diseases, or using any medication that could interfere in the physiological responses of testing or training. Furthermore, the discontinuity criteria were: less than 85% of attendance at the training sessions and/or more than two consecutive missed sessions. Additionally, only subjects with full data available were included in the final analysis.

In summary, after advertising on the university campus and on the local media, 269 obese subjects underwent the initial interview, of which 215 were excluded or disapproved in the clinical evaluation or ECG. Thus, 54 participants were approved and randomly assigned in combined training group (CTG) or control group (CG). However, given the samples available for total ghrelin analyses, only 22 subjects distributed between the CTG (n=12) and the CG (n=10) were included in the final analysis of this study.

All participants were informed about the purpose and risks of the study and signed an informed consent document. The experimental protocol adhered to the declaration of Helsinki and was approved by the research ethics committee of the local university (protocol No. 1278/2011).

Study Design

The CTG performed 24 weeks of CT, while the CG individuals were instructed to maintain their lifestyle. Both groups were instructed to maintain their physical activity pattern and dietary intake during the study. Before the beginning of the study, all subjects for both groups (GC and CTG) were familiarized with the testing equipment and the CTG, as well as with the training protocol. The anthropometric measurements, cardiorespiratory test, maximal strength assessment and plasma concentrations of ghrelin and TNF-α were determined before (Pre) and after 24 weeks (Post) of the experimental period. All assessments were conducted during the same period of the day, with controlled conditions of temperature (22°C) and relative humidity.

Anthropometric evaluations

The same investigator performed all the anthropometric assessments. Weight was taken using a calibrated manual scale (Filizola, São Paulo / Brazil) and height was measured using a wall mounted stadiometer (precision of 0.1 cm). BMI was calculated by dividing body mass (kg) by height squared (m²). Waist circumference was measured midway between the lowest rib and the iliac crest. Subcutaneous skinfold thickness was measured in triplicate at the chest, abdomen and thigh using a skinfold caliper (LANGE®, Cambridge, Maryland / EUA) and standard technique. Body density and percentage body fat (BF) were estimated using the Jackson and Pollock30 and Siri equations31, respectively.

Cardiorespiratory assessment

All the cardiorespiratory tests followed the same procedures carried out in previous studies performed in our laboratory21,32. The subjects performed a maximum effort protocol on a Quinton TM55 treadmill (Bothell, Washington / EUA) where gas exchange was continuously collected using an automated breath-by-breath metabolic cart (CPX, Medical Graphics, St. Paul, Minnesota / USA). The protocol consisted of an initial warm-up of 2 minutes at 4km/h, rising in increments of 0.3 km/h each 30 seconds until exhaustion, followed by a 5-minute recovery period. A 1% gradient13 was maintained until exhaustion and released in the recovery period. The mean of highest 30-s value of oxygen consumption was expressed as the peak oxygen consumption (VO2peak). The ventilatory threshold (VT) and respiratory compensation point (RCP) were determined using visual graphic analysis by three experienced researchers familiar with the CPX Medical Graphics system.

Maximal-strength assessments

Maximal-strength was measured by a one-repetition maximum (1RM) test on bench press, leg press and arm curl exercises performed on RIGUETTO® equipment (São Paulo / Brazil) according to descriptions by Brown and Weir14. The subjects were required to perform 10 repetitions at 50% of their estimated 1RM. After 3 minutes of rest, they were required to perform three repetitions at 70% of their estimated 1RM. After another 3 minutes of rest, subsequent trials were performed for 1RM with progressively heavier weights until the 1RM was determined in up to three attempts, with 3–5 minutes of rest between trials.
**Dietary Intake**

All participants were informed about the importance of maintaining their previous nutritional patterns during the study. They were instructed by trained nutritionists to complete food records for three nonconsecutive days (two days in the week and one day at the weekend) in the first and last week of the study period. The mean of the three food records was used as the dietary intake of each subject. Total calories, carbohydrates, lipids and protein were calculated using the DietPro® Software program, version 5i (Vicosa, Minas Gerais / Brazil).

**Blood Sampling and Biochemical Analysis**

Approximately 10 ml of blood was collected from the antecubital into Vacutainer® tubes (Becton Dickinson Ltd, Oxford/England) for plasma samples (containing anticoagulant EDTA), in the morning (07:00 – 09:00 a.m.), after a 12 hour overnight fast and 72 hours after the last training session or evaluation. All samples were collected, processed, divided into aliquots, and stored at -80°C for subsequent analysis.

Total ghrelin and TNF-α levels were determined in duplicate by enzyme-linked immunosorbent assay (ELISA), according to the specifications of the manufacturer Millipore Corporation® (Billerica / USA), for Ghrelin; and Quantikine high sensitivity kit, R&D Systems®, (Minneapolis / USA), for TNF-α. The sensitivity, intra-assay and inter-assay coefficient of variation for ghrelin were 100 pg/ml, 1.26% and 7.81%, respectively. The sensitivity, intra-assay and inter-assay coefficient of variation for TNF-α were 0.106 pg/ml, 8.7% and 10.4%, respectively.

**Training protocol**

The CT protocol was the same as described in Brunelli et al.21 and consisted of RT and AT performed in the same session, three times per week on alternate days, divided into three stages (S1, S2 and S3) with different intensities at each stage. In S1, RT comprised six exercises (bench press, leg press, pulley, leg extension, arm curl, leg curl) and the subjects performed 3 sets of 10 RM with a maximum of 60 seconds of rest between sets and exercises35. After this, participants were taken to an athletic track and performed 30 minutes of walking or running with varying intensity (5 minutes at less than VT, 10 minutes at VT, 10 minutes between VT and RCP, 5 minutes at less than VT), with intensities corresponding to 50-85% of VO2peak.

In S2, RT was performed with the same exercises from S1, however, the subjects performed 3 sets of 8 RM with 1 minute and 30 seconds of rest between sets and exercises35. In the AT, the same duration was maintained and a new adjustment in the training zone was applied (3 minutes at VT, 12 minutes between VT and RCP, 10 minutes at RCP and 5 minutes at VT), with intensities corresponding to 50-85% of VO2peak.

In S3, RT was performed with the same exercises from S1 and S2, however the subjects performed 3 sets of 6 RM with 1 minute and 30 seconds of rest between sets and exercises35. In the AT, the same duration was maintained and a new adjustment in the training zone was applied (3 minutes at VT, 12 minutes between VT and RCP, 10 minutes at RCP and 5 minutes at VT), with intensities corresponding to 50-85% of VO2peak.

**Statistical Analysis**

Data distribution was tested by the Shapiro-Wilk test. The Student t-test was used to verify differences between groups at baseline and for the magnitude of changes (Δ%) of Ghrelin and TNF-α. A mixed model ANOVA for repeated measurements was applied to compare groups (CG and CTG) and times (pre and post) for all variables, except Ghrelin level. There were differences or a trend towards differences in the baseline values of Ghrelin. Thus, these data were analyzed by the ANCOVA test using the pre values as covariate. When appropriated, the Tukey post hoc test was performed to localize differences. The association between Ghrelin, TNF-α and other variables used in the present study were tested by Pearson’s correlation test. The data were analyzed using the SAS® 9.2 software package. The level of significance was set at p ≤ 0.05. All data are presented in values of mean ± SD.

**Results**

**Baseline Variables**

There were no significant differences between groups as regards body composition, functional capabilities, dietary intake and serum concentrations of TNF-α at baseline (p > 0.05). However, a tendency of difference for the concentration of ghrelin (p = 0.07) was observed and because of this an ANCOVA analysis was performed.

**Anthropometric measurements and Functional Capabilities**

Table 1 presents the results for anthropometric measurements and functional capabilities tests. Decreases in body fat percentage and waist circumference for CTG at the Post moment as compared to the Pre moment (p = 0.001; p = 0.001, respectively) were found. In addition, fat free mass demonstrated increases for CTG at the Post moment as compared to the Pre moment (p = 0.001). Furthermore, increases were found in waist circumference for CG at the Post moment as compared to the Pre moment (p = 0.001). There was no significant difference in body weight during the study (p > 0.05; Table 1).
There were significant increases in 1RM bench press, leg press and arm curl for CTG at the Post moment as compared to the Pre moment (p < 0.0001; p < 0.0001, p < 0.0001, respectively). In addition, 1RM bench press demonstrated decreased for CG at the Post moment as compared to the Pre moment (p = 0.002). Furthermore, a significant difference between the groups was found for 1RM bench press at the Post moment (p = 0.01; Table 1).

There was a significant increase in VO\textsubscript{2peak} for CTG at the Post moment as compared to the Pre moment (p < 0.001; Table 1).

**Dietary Intake**

Table 2 presents the results for dietary intake assessments. There were no significant differences in carbohydrates, lipids, protein and total calorie ingestion both within and between groups (p > 0.05; Table 2).

**Ghrelin and TNF-α**

There were no significant differences in total ghrelin levels for both CG and CTG groups after the experimental period (p > 0.05; Figure 1).

Additionally, there was no significant difference in TNF-α level for CTG after the training period (p > 0.05; Figure 2). However, a significantly increase in TNF-α for CG at the Post moment as compared to the Pre moment was found (p = 0.05; Figure 2).

Furthermore, Pearson’s test did not show any correlation between ghrelin and TNF-α changes (%) for CTG (r = 0.03, p = 0.91) and CG (r = 0.25, p = 0.48).

Table 1. Anthropometric measurements and functional capabilities before (Pre) and after (Post) 24 weeks of combined training in obese middle-aged men.

<table>
<thead>
<tr>
<th>Variables</th>
<th>CG</th>
<th>CTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>49.1 ± 5.5</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>93.7 ± 9.0</td>
<td>94.6 ± 9.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.0</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>30.9 ± 1.5</td>
<td>31.2 ± 1.9</td>
</tr>
<tr>
<td>BF (%)</td>
<td>32.7 ± 5.4</td>
<td>33.7 ± 6.1</td>
</tr>
<tr>
<td>FFM (%)</td>
<td>67.3 ± 5.4</td>
<td>69.7 ± 12.1</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>100.8 ± 3.9</td>
<td>103.6 ± 5.3*</td>
</tr>
<tr>
<td>VO\textsubscript{2peak} (ml/Kg/min)</td>
<td>28.5 ± 4.8</td>
<td>28.9 ± 4.2</td>
</tr>
<tr>
<td>1-RM Leg Press (kg)</td>
<td>315.6 ± 73.3</td>
<td>324.4 ± 77.3</td>
</tr>
<tr>
<td>1-RM Bench Press (kg)</td>
<td>71.0 ± 14.8</td>
<td>60.1 ± 14.1*</td>
</tr>
<tr>
<td>1-RM Arm Curl (kg)</td>
<td>29.9 ± 4.9</td>
<td>30.8 ± 6.3</td>
</tr>
</tbody>
</table>

CG – Control group; CTG - Combined training group; BMI - body mass index; BF - body fat; FFM – fat free mass; WC - waist circumference; VO\textsubscript{2peak} - peak oxygen consumption; 1-RM – one repetition maximum. *Significantly different from Pre; #Significantly different from CG; p ≤ 0.05.

Table 2. Carbohydrates, protein, lipids and total calories ingestion before (Pre) and after (Post) 24 weeks of combined training in obese middle-aged men.

<table>
<thead>
<tr>
<th>Variables</th>
<th>CG</th>
<th>CTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>CHO (g)</td>
<td>236.9 ± 127.8</td>
<td>254.1 ± 131.1</td>
</tr>
<tr>
<td>PRO (g)</td>
<td>92.4 ± 52.5</td>
<td>93.1 ± 49.3</td>
</tr>
<tr>
<td>LIP (g)</td>
<td>91.2 ± 49.9</td>
<td>97.7 ± 58.8</td>
</tr>
<tr>
<td>Total calories (kcal)</td>
<td>2206.8 ± 1193.2</td>
<td>2284.7 ± 1240.9</td>
</tr>
</tbody>
</table>

CG – Control group; CTG - Combined training group; CHO – Carbohydrate; PRO – Protein; LIP –Lipids; p ≤ 0.05.
Figure 1. (A) Total ghrelin levels and (B) magnitude of changes in total ghrelin (Δ%) before (Pre) and after (Post) 24 weeks of combined training in obese middle-aged men. CG – Control group; CTG – Combined Training Group; p ≤ 0.05.

Figure 2. (A) Tumor necrosis factor-α (TNF-α) levels and (B) magnitude of change in TNF-α (Δ%) before (Pre) and after (Post) 24 weeks of combined training in obese middle-aged men. CG – Control group; CTG – Combined Training Group. * Significantly different from Pre; p ≤ 0.05.
Discussion

This longitudinal study investigated changes in total ghrelin levels and their relation to anti-inflammatory effects of exercise training following 24 weeks of CT in obese middle-aged men. We hypothesized that modulations in total ghrelin levels induced by CT might influence the reduction of TNF-α levels in obese individuals, since ghrelin exerts an anti-inflammatory effect by negative modulation on the production of pro-inflammatory cytokines. Contrary to our hypothesis, total ghrelin levels did not increase in response to CT and were not correlated with changes in TNF-α. However, the exercise training was able to improve body composition and functional capabilities and contained the worsening of systemic inflammation associated to obesity.

Although several studies have investigated AT and RT as a method to modulate total ghrelin, few studies have used CT. In the present study, we did not observe changes in total ghrelin and body weight after 24 weeks of CT in obese middle-aged men, even with changes in body fat. Our results were similar to the study of Kadoglou et al., where the authors also did not find increases in total ghrelin and decreases in body weight after 24 weeks of AT, RT or CT in obese diabetic middle-aged individuals. On the other hand, Markofski et al. found significant increases (40%) in total ghrelin and no changes for body weight following 12 weeks of CT in older adults. Thus, it seems that the age and the amount of body fat before the intervention period could influence the effects of a CT program on participants.

Furthermore, the lack of alterations in body weight in both the present study and the study of Kadoglou et al. could be one of the reasons why CT did not promote increases in total ghrelin after the experimental period. This is in accordance with Leidy et al., where the authors observed that women who lost weight revealed greater ghrelin increases as compared to those who did not lose weight after a 3-month AT protocol. In another study, Foster-Schubert et al. found increases in total ghrelin levels and reductions in total weight after a 1-year AT intervention in postmenopausal overweight women. Whatsmore, Mager et al. and Kadoglou et al. did not find significant changes for ghrelin and total weight after 36 or 24 weeks of RT, respectively, in middle-aged individuals.

Curiously, one result found in the present study was the increase in TNF-α levels for CG after the experimental period. In addition, CG demonstrated a significant rise in waist circumference in the Post 24 weeks when compared to baseline values, even with no change in food intake during the experimental period. In a study by Gradmark et al., the authors demonstrated that visceral adiposity can be assessed using waist circumference with high correlation with computerized tomography. Thus, if it is well known that elevated visceral adipose tissue is a major source of pro-inflammatory molecules that contribute to low-grade chronic inflammation associated with obesity, our results suggest a worsening of systemic inflammation associated with inactive lifestyle and obesity in the CG after the 24 weeks. Furthermore, the CT protocol was effective in containing the worsening in the chronic systemic-inflammation associated with obesity in the CTG.

Although the CT protocol in the present study was not effective in promoting changes in body weight and total ghrelin, this protocol was able to improve body composition through the reduction of waist circumference, fat mass percentage and increase in lean mass, and also improved functional capabilities as evidenced by an increase in VO_{peak} and general strength. Additionally, the protocol was efficient in containing the worsening of systemic inflammation evidenced by the maintenance of TNF-α levels in the CTG, in contrast to those observed in CG that showed worsening of the inflammation after the trial period represented by the significant increase in TNF-α levels.

Contrary to our hypothesis that ghrelin modulation would be related to TNF-α change, the changes of these markers in both CTG and CG were not correlated. Although this correlation is presented in other studies, suggesting the anti-inflammatory role of ghrelin, these analyses are often performed with in vitro or animal models differing substantially from our groups of obese middle-aged individuals. As regards exercise training, few studies have found the correlation between changes in ghrelin and inflammatory markers without associated weight loss. The majority of studies without weight loss fail to show association between these markers, including ours. Thus, we suggest that changes in ghrelin did not contribute to the anti-inflammatory effect of exercise in the present study, since the same CT protocol used in our previous study demonstrated significant reductions in the inflammatory markers associated with chronic low-grade inflammation linked to obesity, even with no changes in weight.

It is important to state that this study is not without limitations. One important limitation in the present study is that we measured total ghrelin levels only, which gives the resulting levels of both acylated and unacylated ghrelin. Future research should examine the effects of prolonged CT in acylated and unacylated ghrelin in order to reveals more information about exercise training effects on these peptides. Thus, from the point of view of practical implications from the present study, CT may bring benefits to the obese population such as improvement of body composition and functional capabilities as well as containing the systemic inflammation in this population.

Conclusions

In conclusion, 24 weeks of CT without diet intervention did not promote changes in total ghrelin and TNF-α levels in obese middle-aged individuals. This may be due to the lack of alterations in body weight after the experimental period, since ghrelin circulates in proportion to body weight (with higher levels of ghrelin seen with lower body weight) and generally responds in a compensatory fashion to weight change (increasing with weight loss, decreasing with weight gain). However, this protocol was effective in improving body composition and functional capabilities and containing the worsening of systemic inflammation as evidenced by the maintenance of TNF-α levels in the CTG after the experimental period. In addition, modulations in ghrelin levels appear not to be associated with systemic inflammation as measured by TNF-α.
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