Ghrelin Plasma Levels and Gastric Tissues Expression in Patients Submitted to Laparoscopic Sleeve Gastrectomy as Primary or Revisional Weight Loss Procedure

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Abstract

Introduction: Ghrelin (Ghr) plays a role in the regulation of food intake and Laparoscopic Sleeve gastrectomy (LSG) is used for treatment of morbid obesity (MO) and after this the expression of ghrelin could be modulate. The aim of present study was to analyze the expression of ghr in three areas of resected stomach specimens from MO patients and co relate these data with plasmatic ghrelin levels before and after surgery.

Materials and Methods: 36 morbidly obese patients (17%, 6/36 with Type 2 Diabetes) were subjected to LSG and tissue samples were obtained from the fundus, body, prepyloric of the resected stomach. For mRNA and protein expression analysis. Blood samples were collected before and 1 month after surgery to evaluate the plasmatic ghrelin levels.

Results: Ghrelin protein expression was higher in the fundus than in the other areas. T2DM patients showed a lower basal ghrelin plasma level compared with non-diabetic patients but they showed a high percentage of positive cells in the stomach. Was not observed a statistical difference in plasmatic, mRNA and protein expression of ghrelin between primary LSG patients and in revisional LSG group.

Conclusion: Ghrelin fundal mucosal expression was comparable in primary and revisional LSG. Diabetic patients showed a compensatory higher protein mucosal expression probably to balance lower plasma Ghrelin level. Further studies will elucidate the clinical relevance of those preliminary data.

Keywords: Ghrelin; Laparoscopic sleeve gastrectomy; Morbid obesity

Introduction

Ghrelin (Ghr), a ligand for the growth hormone secretagogue receptor, is an orexigenic hormone produced primarily by cells in the oxyntic glands of the stomach [1-4]. It is known to increase appetite and regulate weight and body composition [5]; it plays a physiological role in the regulation of food intake (meal initiator) with plasma ghrelin levels rising shortly before meals and declining rapidly after food intake in humans and rodents [3,6,7]. Although produced throughout the length of the intestine, ghrelin is mainly secreted by the stomach, followed by the small intestine but it is also expressed by peripheral tissues, such as pancreas and brain [1,8,9].

Interestingly, fasting plasma ghrelin concentration is negatively correlated with Body Mass Index (BMI), so the ghcr concentration is lower in obese patients than in lean subject [7,10]. Plasma ghrelin levels increased in patients with eating disorder, like anorexia nervosa and cachexia [11-13]. The regulatory mechanism of ghrelin expression is not completely understood and moreover the plasmatic ghrelin levels are affected by several conditions like pre-analytic factors in plasmatic ghrelin analysis, stress, diet and its circadian rhythm [5].

Laparoscopic Sleeve Gastrectomy (LSG) is rapidly gaining acceptance both as a first stage of bilipancreatic diversion with duodenal switch (BPD-DS) or bridge procedure to gastric bypass in high risk patients or definitive procedure for treatment of morbid obesity (MO) [14,15]. Recently, LSG has been proposed as an alternative revisional procedure for failed or complicated gastric banding [16]. Most bariatric surgeons perform the revisional procedure in one stage and the outcomes in terms of weight loss seem to be comparable with the primary LSG [17-22]. In order to reduce perioperative complications rate, some authors have proposed to perform the LSG in “two stage” after the band removal [23-25]. The main steps of the procedure includes a vertical resection of most of the stomach volume calibrated on an orogastric bougie, removing part of the antrum, most of the body, and all of the fundus [14,26]. The proposed mechanism of action of this procedure is a combination of volume restriction, creation of a high pressure system and the induction of a favourable hormonal change [27,28]. Therefore crucial points of the technique are the amount of antrum resected (distance of the stapler line from the pylorus), orogastric bougie size and completed fundus dissection to allow a proper total fundectomy. The left crus of the diaphragm should be systematically exposed and the posterior attachment of the fundus released [27]. Since LSG removes the area of stomach producing ghr, low plasma ghrelin levels were monitored up to 5 years [15].

MO individuals report a decline in appetite after LSG, presumably due, in part, to ghcr cell removal [1]. Moreover, there are strong evidence that surgery can “heal” most of the MO patients with type 2 diabetes mellitus (T2DM) diagnosed less 10 years but the mechanism is not completely understood [29-32].
Recently, some authors have studied the localization of ghr expression to try to clarify the clinical significance of ghr in obese patient's submitted to LSG [5,33].

Aim of this prospective study was to evaluate ghr protein tissue distribution in different stomach areas by immunohistochemical analysis and mRNA gene expression by Real-Time Polymerase chain reaction (RT-PCR) on fresh stomach specimens tissue of LSG carried out as primary or revisional intent. Another important end point was to analyze the circulating plasma ghr levels before and 1 month after surgery and to correlate these values with mRNA and protein ghr expression.

Materials and Methods

Population study

Thirty-six MO patients, 6 men and 30 women with median age of 39 years old (range 22 to 61 years) consecutively underwent LSG, has been enrolled prospectively. Exclusion criteria were: not compensated chronic liver or renal disease; BMI >60 Kg/m², procedure intent as first stage of BPD-DS, conversion to open surgery, age <18 and >65 years old. Median pre-sleeve BMI value was 44.5 Kg/m² (range 36-57) and 9 (25%) patients underwent LSG as revisional procedure after gastric banding failure (mean interval band removal-sleeve was 5 months). Six patients (17%), all women with median age of 52 years old, presented 9 (25%) patients underwent LSG as revisional procedure after gastric banding failure. Gagner [23]. The division of the gastric greater curvature vascular supply started 6 cm from the pylorus (antrum preserving procedure) and proceeded upwards to the angle of His; ultrasound dissection or radiofrequency were used. The gastro-esophageal junction was always assessed, and a hiatal hernia, if found, was repaired. The LSG was proceeded using a linear stapler. The stapler was applied next to a 42 Fr blue test was performed and a 19 Fr drain was placed alongside the transected stomach. The stomach was removed throughout a 12 mm port incision enlarged to avoid specimen damage. A stitch was put on the distal resection margin (pre-pyloric area).

Plasma ghrelin determination

The plasmatic levels of ghr were measured by a commercial radioimmunoassay kit (LINCO Cat# GHRT-89HK). In all fasting overnight patients a draw of peripheral blood was obtained the day of surgery and 1 month after surgery. Radioactivity was determined by the WALLAC gamma counter (1261). Two samples were excluded due to technical error in sample handling.

Immunohistochemical analysis

Stomach specimens resected during SG were fixed in buffered formalin 10% for 24 hours. After fixation, three tissue samples were obtained from 3 areas of each stomach: the fundus, body and pre-pyloric area.

The avidin–biotin complex (ABC) method was performed on 4 μm thick tissue sections for immunohistochemical analysis. Sections were deparaffinized with xylene for 15 min and were treated in a microwave oven using 0.01 M citrate buffer (pH 6.0) for 30 min. The mouse IgG monoclonal antibody directed against ghrelin (Neomarker, Fremont, CA, USA) was used. The reaction was performed in an automated system (Bond Max, Menarini, Italy). Results were expressed according to a semiquantitative analysis: two pathologists evaluated the percentage of cells positive in 3 different areas (20X, fundus, body, pre-pyloric) of each sample; the mean value of the three different areas was used to define the value of positive cells in each sample. For each patient, the ghr expression was calculated using the mean value of positive cells of three areas.

Real-Time Polymerase Chain reaction

RNA from frozen tissue was extracted from fundus of the stomach using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the supplier’s instructions, which provided an elution in a final volume of 100 μL. The extracted RNA was quantitated by OD260/280 measurement; next total RNA was reverse-transcribed in a final volume of 20μL using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA). The cDNA was stored at -20°C until it was used.

Then, 30 ng cDNA was added to 2X TaqMan Fast Universal PCR Master Mix (Life Technologies, Foster City, CA, USA) in a final volume of the 10 μL. Real-Time quantitative PCR for ghr mRNA was performed on an ABI PRISM 7500 Fast Real Time PCR System (Life Technologies, Foster City, CA, USA). Amplification of cDNA was performed under the following conditions: 20s at 95°C, 3s at 95°C and 30s at 60°C for 40 cycles. PCR products for ghr were detected using gene-specific primers and probes labeled with reporter dye FAM (Ghr gene ID: 51738, Life Technologies, Foster City, CA, USA). GAPDH was selected as endogenous control and the expression of ghrelin was compared to this housekeeping gene; GAPDH was detected using gene-specific primers and probes labeled with reporter dye FAM (gene ID: 2597, Life Technologies, Foster City, CA, USA). Amplification products length of ghr and GAPDH was 62 and 58 base pairs respectively. PCR reaction was carried out in triplicate on 96-well plate, at the end of the reaction; the results were evaluated using the ABI PRISM 7500 software. The Ct (Cycle threshold) values for each set of three reactions were averaged for all subsequent calculations.

Statistical analysis

All data obtained from the various analyses will be collected in a customizable database built with Microsoft Access (Microsoft Corporation). Relevant data will be extracted from the database with appropriate queries and exported in Microsoft Excel (Microsoft Corporation) and SSPS v.08 for further manipulations and statistical analyses.

Results

Plasmatc ghrelin levels

Total ghr plasma levels decreased from 69.2 ± 99.4 pg/mL before surgery to 14.4 ± 31.6 pg/mL after surgery (Figure 1). In T2DM patients the ghr level before surgery was 40.7 ± 44.5 pg/mL while in non-T2DM patients was 75.1 ± 127.2 pg/mL; one month after surgery, ghr levels in
T2DM and non-T2DM patients were 21.5 ± 31.6 pg/mL and 12.7 ± 27.2 pg/mL respectively.

Was not observed a statistically significant difference in the values of plasma ghrelin before surgery between patients undergone LSG as primary procedure versus those subjected to revisional (two stages approach) LSG, which showed 73.9 ± 127.2 pg/mL and 55.5 ± 99.4 pg/mL respectively; a statistically difference was not observed in the plasma ghrelin values one month after surgery too (16.1 ± 31.6 pg/mL and 5.7 ± 17.2 pg/mL).

Mean BMI was 44.5 ± 10.5 kg/m²; a negative correlation between the basal plasma ghrelin levels and BMI was observed (p=0.01). T2DM patients showed basal plasmatic ghrelin value lower than non-T2DM patients (40.7 pg/mL vs. 75.1 pg/mL, p=0.05, Table 1).

**Immunohistochemical results**

Immunohistochemical expression of ghrelin was detected in 25.0% of gastric cells; furthermore, we observed that ghrelin-producing cells were distributed differently in investigated areas. Indeed, the prevalence of ghrelin-producing cells was higher in the fundus than in the body and even less in the pre-pyloric area near the distal resection surgical margin. The average ghrelin cells counts were 27.7 ± 45.6, 23.9 ± 29.3, 23.6 ± 28.0 cells/field in the fundus, body and pre-pyloric area respectively (Figures 2 and 3) but this trend was not statistically significant. In T2DM patients the immunohistochemical expression of ghrelin was 30.7% while in non-T2DM patients was 23.9% (p=0.02). The ghrelin immunohistochemical expression was similar in primary and revisional sleeve (25.0% and 25.1%). We didn’t find correlation between protein expression and ghrelin plasma level.

**Quantitative mRNA ghrelin expression**

Level of ghrelin mRNA expression was normalized to the expression of GAPDH; the mean value of RQ (Relative Quantification) of ghrelin mRNA expression was 2.6 ± 12.5. The T2DM patients showed a value of ghrelin mRNA expression of 2.1 ± 2.2 while non-T2DM patients showed a mean value of 2.6 ± 12.3. Ghr expression was significantly higher (3.0 ± 12.2) in primary LSG group than in revisional 1.2 ± 2.0 (p<0.05).

We find a strong correlation between the mRNA expression and the protein expression of ghrelin in the fundus (p=0.0001), while we found a weak correlation between the mean value of mRNA expression and the mean value of ghrelin protein expression obtained from the three areas (fundus, body, pre-pyloric, p=0.04). Also in this case, we didn’t find correlation between mRNA expression and plasmatic levels of ghrelin.

**Discussion**

The decrease of plasma ghrelin after LSG is advocated as one of the hormonal mediator of the weight loss as well as glucose homeostasis in the early phase in absence of a significant weight loss. Several experimental and clinical studies suggest a change of incretin levels related to the marked post-LSG ghrelin decrease [35-38].

In this prospective study, ghrelin expression and tissue distribution of ghrelin mRNA expression were compared between T2DM and non-T2DM patients who underwent primary or revisional sleeve gastrectomy. The results showed a significant decrease in plasma ghrelin levels after surgery, with a stronger reduction in T2DM patients. The immunohistochemical analysis revealed a lower prevalence of ghrelin-producing cells in the fundus area, which was consistent with the decrease in ghrelin expression. Quantitative mRNA analysis also supported these findings, with a higher expression of ghrelin mRNA in primary LSG compared to revisional LSG.

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cells producing this protein were evaluated to better understand the ghrelin distribution in different areas of stomach and correlate those findings with ghrelin plasmatic levels in different groups of patients undergone LSG (primary sleeve, revisional sleeve, diabetic).

The total plasma ghrelin level in all study patients before surgery was correlate to BMI as previously reported [10,39]. The ghrelin plasma level significantly decreased after surgery in all study groups as a result of proper total fundectomy [11,15]. On the other hand, several studies don’t find a modification of ghrelin plasma levels and other authors report controversial results including an increment of ghrelin plasma value after surgery [40,41]. The contradictory results could be due to different study designs, follow-up periods, measure methods, surgical intervention and circadian rhythm [5,11].

In agreement with Goiten et al. [33], our immunohistochemical results showed that ghrelin protein expression was higher in the fundus than in the body and pre-pyloric area; although this difference was not statistically significant. Miyazaki et al. hypothesize that greater is the number of positive cells present on the stomach, better will be the surgery outcome and, if the number of positive cells present in the stomach correlates with the mRNA ghrelin expression, this value could be considered a favourable predictor of LSG outcome [5]. We found a distinct correlation between ghrelin mRNA expression and ghrelin protein expression in the fundus and a weak correlation between ghrelin mRNA expression and the mean value of ghrelin protein expression obtained from the three areas (fundus, body, pre-pyloric). Nevertheless, as previously reported [33], levels of ghrelin mRNA didn’t correlate with plasmatic protein levels, this could be due to compensatory productions from extra gastric organs.

Moreover, T2DM patients showed a lower basal (pre-operative) ghrelin plasma level compared with non-diabetic patients. Several studies have found that low ghrelin plasma levels are associated with elevated fasting insulin concentration and the prevalence of T2DM and insulin resistance and levels of plasmatic ghrelin is found to be lower in obese T2DM patients than to equally obese insulin-sensitive controls [42,43]. However, we observed that the percentage of ghrelin positive cells in T2DM patients was significantly higher compared to no-T2DM; this could suggest that T2DM patients developed a hyperplasia to compensate the lack of circulating ghrelin.

Recently, there is a debate on the extension of the antral resection during SG: some authors recommend to preserve the antrum because this site is important as a pumping mechanism for gastric emptying, during SG: some authors recommend to preserve the antrum because the partial antrum resection doesn’t affect significantly the long term pouch volume [44,45] and because the removal of antral tissue could be not the real population of cells producing ghrelin [46,47]. Therefore, we believe that the antral tissue is not a major source of circulating ghrelin as previously reported [48,49]. Some authors argue the cells population present on antral tissue since the majority of positive cells were present on fundus tissue, as previously described [46,47] but this should be confirmed on a larger population.

We didn’t find differences between patients who underwent to primary SG and revisional SG, both in protein expression and mRNA levels and basal plasmatic levels of ghrelin. These findings support the positive short and mid-term results of LSG as revisional procedure compared to primary.

The limits of the present study are: small numbers of diabetic patients and non-comparable size group (primary vs. revisional), short term plasma level measurements, no long term follow up. Further studies are needed to confirm the preliminary results in a large population.

In conclusion, the preliminary results of the on-going prospective study confirm that ghrelin protein expression was higher in the fundus than in the body and pre-pyloric areas. Moreover, T2DM patients showed a lower pre-operative ghrelin plasma level and more ghrelin positive cells in stomach compared with non-diabetic patients. We didn’t found difference in ghrelin plasma level between patients who underwent to primary or revisional SG supporting the clinical evidence of similar excess weight loss. Further evaluation on a large population will confirm or not those preliminary findings.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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