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Pancreas is a preeminent source of ghrelin after sleeve gastrectomy in Wistar rats

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Summary. Many surgical techniques are employed in the treatment of severe obesity. A main consequence of these techniques is the improvement of type 2 Diabetes mellitus. Ghrelin is a gut hormone released in the gastric fundus and corpus, which has been related to diabetic improvement as mentioned in these papers. Sleeve gastrectomy and Roux-en Y Gastric Bypass are surgical techniques broadly employed in humans; both severely reduce the gastric surface. Paradoxically, the serum level of ghrelin in patients is preserved. We hypothesized about the role of embryonic pancreatic epsilon cells, which have the capacity to release ghrelin. We studied the changes in the epsilon cells and differentiation markers with immunostaining and ghrelin serum level and after surgery. We employed euglycemic male Wistar rats: two surgical groups (Sleeve gastrectomy and Roux-en Y Gastric Bypass) and two control groups. We reported a significant increase of ghrelin epsilon-cells in the pancreas and basal serum after Sleeve gastrectomy versus the control groups. The epsilon cellular increment was related to neogenesis, as the neurogenin-3 marker revealed. The Roux-en Y Gastric Bypass showed neither epsilon cell increase nor basal serum changes in ghrelin release. As a conclusion, we reported that the severe suppression of the fundus gastric produced the recovery of ghrelin released by the epsilon cells, which was indicative of an ontogenic embryonic pancreatic function.

Key words: Ghrelin, Sleeve gastrectomy, Cell differentiation, Epsilon cell

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Introduction

Diabetes mellitus is a major health problem in most countries (Tao et al, 2015). Many studies have shown the importance of type 2 Diabetes mellitus (T2DM) control to prevent complications of the disease and reduce mortality (Frühbeck, 2015; Batterham and Cummings, 2016). Bariatric and/or metabolic surgeries are now considered as definitive treatment of T2DM, compared to lifestyle modifications and pharmacotherapy (Dixon et al., 2008; Schauer et al., 2012). The intrinsic physiological mechanisms for this improvement remains unclear. Nevertheless, several hypotheses have been reported, some of them focused on the relationship and participation between certain gut hormones such as ghrelin, which is a gut hormone mainly released in specialized X/A-likecells of the adult stomach (Stengel and Taché, 2012).

Ghrelin has basic and broad functions on many organs and system, such as the central orexigenic effect. In the pancreas, the ghrelin functions are essential not only in endocrine secretion but in Langerhans islet cellularity. Thus, ghrelin modulates insulin secretion and

glucose metabolism. However, this hormone does not directly release insulin (Bando et al., 2012; Méndez-Giménez et al., 2017). This modulation of insulin release is downregulated by a previous GLP-1 signal on β-cells (Damdindorj et al., 2012). In another sense, ghrelin stimulates the survival of β-cells (Granata et al., 2007) and reduces the pancreatic steatosis in β-cells in rats after Sleeve Gastrectomy (Kojima and Kangawa, 2005).

Other intestinal locations of ghrelin release have been noted, such as duodenum, jejunum, ileum or colon (Kojima and Kangawa, 2005; Fakhry et al., 2019). Ghrelin seems not to be restricted in the gastroenteral system, and maintains its functions in other organs, such as the central nervous system or testis (Kheradmand et al., 2009). Also, embryonic epsilon (ε) pancreas cells release Ghrelin during these stages (An et al., 2010). Through the embryonic period, ε cells represent almost 10% of endocrine cells. At these embryonic stages, during the differentiation phases progenitor cells express sequentially some transcription factors, which include the neurogenin-3 as the last factor prior to the ε cells (Edlund, 2002; Prado et al., 2004; Heller et al., 2005). Meanwhile, the ε cells presence is reduced after birth in pancreas islets and X/A like cells increase progressively in the stomach (Collombat et al., 2007).

The most common bariatric procedures are Sleeve Gastrectomy (SG) and Roux-en Y Gastric Bypass (RYGB). The greater curvature and fundus of the stomach are resected in SG. Thus, SG induces weight loss by reducing gastric capacity and changes in hormone secretion patterns (Patrikakos et al., 2011). SG decreases fasting and post-prandial plasma ghrelin compared to pre-surgery, because the main source of ghrelin in adults are cells located in the fundus removed during this procedure (Yousseif et al., 2014; Sista et al., 2016). However, surprisingly, after surgical techniques it was reported that the fasting plasma ghrelin levels had increased in human patients (Dogan et al., 2016).

Throughout RYGB, the stomach receives a severe aggression. RYGB creates a small gastric pouch, which is attached to the mid-jejunum; meanwhile most of the stomach and the proximal jejunum is bypassed to the distal jejunum. The reduction of gastric volume does not include the massive resection of gastric mucosae, but it receives a severe aggression. RYGB surgery sections gastric chambers keeping initially the ghrelin release cellularity. These separated compartments cannot receive the same stimuli to release this hormone. In this sense, the changes in ghrelin had been related to food repletion. Ghrelin cell production is conserved after RYGB, as some authors reported (Fonseca et al., 2018; Svane et al., 2019; Xu et al., 2019).

These data lead us to ask for an alternative source of ghrelin after SG or RYGB. Ontogenicaly, the epsilon (ε) ghrelin-producing cells appear during embryonic pancreas development (Collombat et al., 2007). These ε-cells are derived from Neurogenin3-expressing progenitor cells. The ε-cells represent approximately 5-10% of the total number of endocrine islet cells at birth. In the adult stage, the pancreatic ε-cell population declines (Wierup et al., 2004; Andralojc et al., 2009). In adults, the gastric endocrine X/A-Like cell population remains as the major source of circulating ghrelin (Ariyasu et al., 2001). Previous papers had reported that ghrelin could be expressed in human α-glucagon or β-insulin-secreting cells in rats (Raghay et al., 2013). This paracrine action may be related to ghrelin control in islet function and cellularity (Date et al., 2002).

The aim of this study is to analyze endocrine pancreas capability to became an alternative source of ghrelin after bariatric surgery. This remaining ε-ghrelin-producing cell population could proceed from differentiated insulin or glucagon producing cells. The stressful homeostatic situation established after SG or RYGB could expand the ε-epsilon cell mass. Then, this ε-epsilon cell increase could be related to cellular mechanism of proliferation and/or neogenesis. To this purpose we employed Wistar normoglycemic and normal-weighted rats to reproduce these surgical techniques without the bias of pathological conditions, such as obesity of diabetes.

Materials and methods

Animals

Surgical procedures and animal sacrifice technique were performed with approval of University of Cadiz Committee for Ethical Use and Care of experimental animals. This Committee controlled that the procedures in all experiments were performed in accordance with international relevant guidelines and regulations of animal welfare. This Committee is under the control of the Andalusian Authority. The animals were provided by the Animal Service and Production Unit (SEPA, at the University of Cádiz). The male Wistar rats (n=24) were kept in a controlled-environment room (21°C, 12 h/12 h light-dark cycle), with standard laboratory rat chow available ad libitum. To avoid the cyclic variations of gonadotropin hormonal effect on the glycaemic metabolism female rats were not used.

Wistar rats weighed approximately 250 g at the beginning of the study and they were nine weeks old. The rodents were randomly grouped into two surgical groups (RYGB and SG) and two control groups (a fasting group -FC- and a surgical control -Sham-).

Surgical procedures

The fasting group (FC) animals (n=6) suffered the same perioperative fasting periods, related to the surgical protocol. The animals finished an 18 h presurgical and a 12 h postsurgical fasting period. An intake re-adaptation period followed each surgery to normalize fasting and this stage was established for FC and the surgical groups.

All surgical procedures (Fig. 1) were performed in anesthetized animals with continuous infusion of

Isofluorane 3% V/V (Isoflo, Abbott 571329.8). The Sham-technique (Sham) (n=6) reproduced the surgical aggression over the digestive tract. However, the Sham group maintained the integrity of the digestive tube. Sham was performed by an incision of about 3 cm in the middle area of the abdomen, exposing the small bowel loops. After we measured the size from the angle of Treitz to ileocecal valve, a transversal enterotomy section was performed. Without intestinal resection, an end-to-end anastomosis was done.

After abdominal midline incision, Roux-en Y Gastric Bypass (RYGB) group (n=6) underwent laparotomy and a transverse section of the stomach was performed, from the upper pole (rumen) to the minor curvature. This section was closed by continuous suture (Prolene® 4/0), leaving the last 0.5-0.7 cm of the stomach to restore continuity to the food handle. The RYGB, - mixed malabsorptive and restrictive- bariatric surgery involved the exclusion of the proximal intestine by the bypass of the duodenum and a part of the jejunum, as well as the reduction of stomach to the fore-stomach. The jejunum was dissected at 8 cm from the ligament of Treitz, and the terminal jejunum of the section was connected via end-to-end anastomosis to the preserved gastric fold. The antro-jejunal loop (biliopancreatic loop) was continued with the alimentary loop at 10 cm of the fundus-jejunum anastomosis.

The Sleeve Gastrectomy (SG) group (n=6) was performed by a laparotomy of 5 cm in the upper third of the abdomen, through sectioning of the gastrosplenic ligament and exposing the stomach. A curved forceps was applied from the angle of Hiss to antrum to guide the stomach portion to be resected. The continuous suture (Prolene® 4/0) performed a remnant cylindrical stomach of approximately 0.5 cm of diameter. The portion of sectioned stomach included the most fundus, stomach-corpus at greater curvature and antrum. The pylorus was preserved. The SG reduced the initial stomach volume by approximately 20%. SG reproduced the actual selective technique used in humans as the restrictive model of bariatric surgery.

Ghrelin basal serum levels

From the first to thirty-fifth day after surgery, plasma ghrelin concentration was measured in fasting rats of every group. Blood samples were obtained after a fasting period of 12 h from the rat tail. Blood samples were added to EDTA tubes, centrifuged at 4000x g for 15 min at 4°C, plasma removed and frozen at -80°C. Total ghrelin was assessed by ELISA kits (EZRGRT-91K Millipore-Merck Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The intra-assay coefficient of variation was 5,8% and the inter-assay coefficient of variation was 6,2%. Results were expressed as ghrelin fmol/plasma ml.

Tissue preparation

For the next three months, animals were fed with regular chow. The animals were sacrificed after survival period by Isoflurane inhalation overdose. Pancreas were immediately removed and fixed in Bouin's solution overnight at 4°C, dehydrated, embedded in paraffin and cut into serial 10 µm -microtome sections for immunostaining.

Immunostaining analyses

E-cell population was calculated by double immunostaining using guinea pig anti-insulin (Sigma-Aldrich St Louis MO USA) and mouse anti-ghrelin (Abcam, ab209790, Cambridge, UK) antibodies. Fluorescent secondary antibodies were Alexa (Alexa 546 and 488, Molecular Probes Inc. Eugene, OR USA). Islet areas were quantified in 30 islets per condition. Results were noted under randomized conditions by a single investigator and expressed as number of ghrelin positive cells/mm^2 of islet.

E-cell proliferation was assessed by immunofluorescence using mouse anti-PCNA IgG (Abcam, CB4OFL, Cambridge, UK) and rabbit anti-ghrelin IgG (Abcam, ab209790, Cambridge, UK)

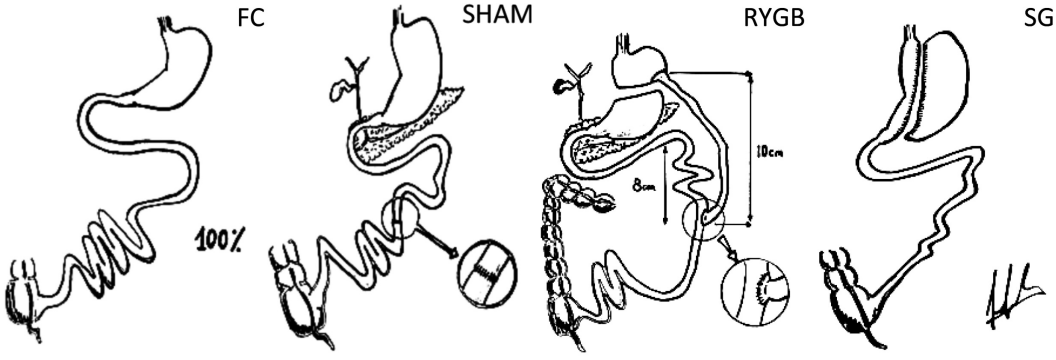

Fig. 1. From left to right, the surgical techniques are presented to facilitate text interpretation: Fasting control (FC) animals did not undergo surgery; Sham group (Sham) an enterotomy without resection was done; Roux en-Y Gastric Bypass (RYGB) and Sleeve gastrectomy (SG) faithfully reproduced the two main bariatric techniques employed in human clinic.

antibodies. Fluorescent secondary antibodies were Alexa (Alexa 546 and 488, Molecular Probes Inc. Eugene, OR USA). DAPI was used to counterstain nuclei. This parameter was quantified in 30 islets per condition. Results were noted under randomized conditions by a single investigator and expressed as number of ghrelin+ cells/PCNA+ cells/mm^2 of pancreas.

E-cell neogenesis was measured through the double staining of Neurogenin-3 marker (NGN3) and ghrelin cells. NGN3 is the late transcription factor in the sequence of new ghrelin cells from progenitor cells. NGN3-positive cells were stained by using rabbit anti-NGN3 antibody (Abcam, 176124, Cambridge, UK) and rabbit anti-ghrelin IgG (Abcam, ab209790, Cambridge, UK) antibodies. This parameter was quantified in 30 islets per condition. Results were noted under randomized conditions and expressed as number of ghrelin+ cells/NGN3+ cells/mm^2 of pancreas.

Also the number of Nkx2.2 positive cells was tested to determine the correct sense of neogenetic cells in every group. Nkx2.2 is a transcription factor present in the differentiation sequence from endocrine progenitor cells to β and α cells, but not to ε-cells. Nkx2.2 positive cells were stained using rabbit anti-Nkx2.2 antibody (Abcam, CB4 OFL, Cambridge, UK). Fluorescent secondary antibody was Alexa 488 (Molecular Probes Inc. Eugene, OR USA) Nkx2.2 positive cell numbers were evaluated in three slices of whole pancreas per surgical condition and expressed as number of Nkx2.2 positive cells/mm^2 of pancreas. Results were noted by a single investigator, under randomized conditions

All immunocytochemical studies were measured using a microscope with digital camera and the image analysis Cell-D software (Olympus, GmbH. Hamburg, Germany).

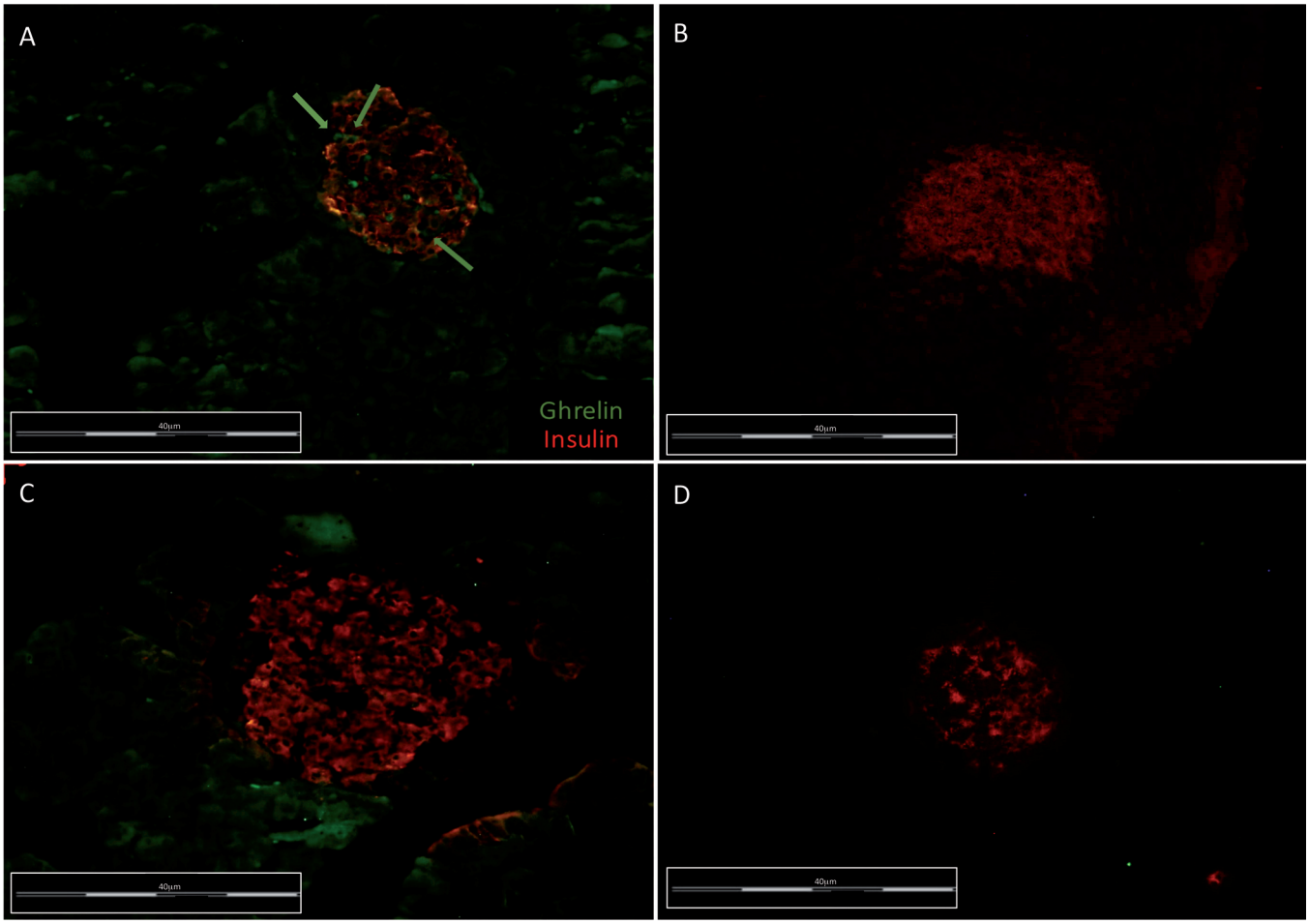

Fig. 2. Representative microphotographs of ghrelin-immunostained pancreas in the experimental groups. The islets show a regular aspect and shape; this usual ovoid aspect is the regular aspect used to present the islets pancreas of Wistar rats specimens. The image shows ghrelin+ cells (in green) expressed in some insulin+ cells (red): **A.** Sleeve Gastrectomy group (SG), an increased number of positive ghrelin-cells (green) appeared in the pancreas of SG Wistar rats compared to the other groups. **B.** Fasting Control (FC). **C.** Sham group. **D.** Roux en-Y Gastric Bypass (RYGB).

Statistical analyses

Results are expressed as means ±SEM of measurements performed. Statistical comparisons were performed either by Mann-Whitney U-test or by ANOVA followed by Sheffé's test. Differences were considered statistically significant at P≤0.05.

Results

Changes of Epsilon cell population

Epsilon cell population was measured twelve weeks after surgery in pancreatic tissue sections using a mouse anti-ghrelin antibody. Values were expressed as the number of ghrelin positive cell/mm^2 of islet area (Fig. 2). Data showed an increased ε-cell population in SG compared with FC or Sham groups but not in RYGB (P<0.01) (Fig. 3). No differences were found between control groups.

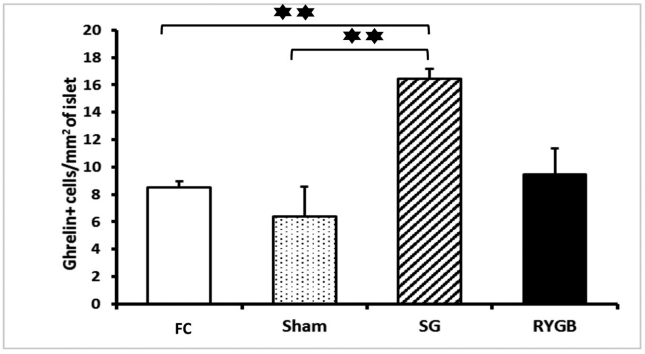

Fig. 3. Ghrelin positive cells (E-cells) presented as ghrelin positive cell /mm^2 of pancreatic islet in the Y axis. Fasting control rats (n=6) (FC, white bar), Sham rats (n=6, dotted bar), SG rats (n=6, striped bar) and RYGB rats (n=6, black bar) are represented in the X axis. Values were represented as mean ±EEM. (*p<0.05) (**p<0.01). Data obtained are FC: 8.53±0.42; Sham: 6.40±2.15; SG: 16.43±0.42; RYGB: 9.48±1.85.

Epsilon cell basal levels

We studied the ghrelin plasma level in the four groups. The basal serum level of ghrelin can be related to the changes in ε-pancreatic ghrelin-expressing cells. After a 12 h fasting period, the basal serum ghrelin levels revealed different data between both surgical groups versus control groups (Fig. 4). In RYGB group, the ghrelin plasma level showed a permanent decreased plasma level versus control groups (p<0.05). A similar consideration was found in the SG group during the first two weeks after surgery. Then, SG group showed a progressive increase to normal ghrelin plasma level from day 21 to the end of the assay (p<0.05). No significant differences were found between CA or Sham groups at any time.

Epsilon cell proliferation

To clarify the possible cellular mechanism of proliferation in the changes of ε-cell population expansion, pancreas samples from all the groups studied were marked with anti-PCNA antibodies. PCNA is a widely used proliferation marker. No significant differences were found between the groups (Fig. 5).

Epsilon cell neogenesis

Another path for the expansion of ε-cell population can be the increase from stem cell differentiation. To

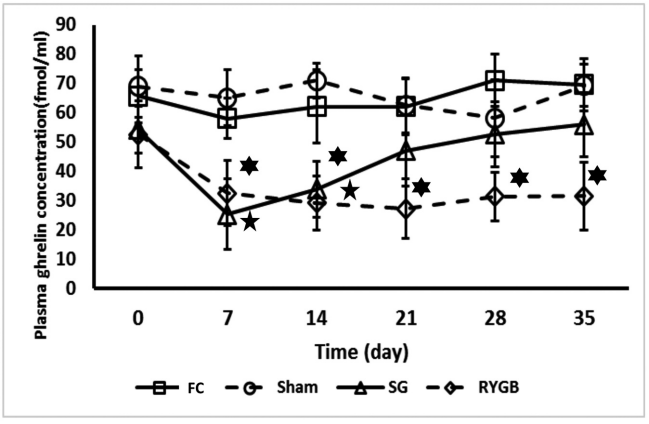

Fig. 4. Fasting ghrelin blood level presented as femtomol/ml in the Y axis for Fasting control (FC, solid black line with squares), Sham (discontinuous black line with circles), SG (solid black line with triangles) and RYGB (discontinuous black line with diamonds) groups are presented along time (days) in the X axis. Values are represented as mean + EEM. (*p<0.05).

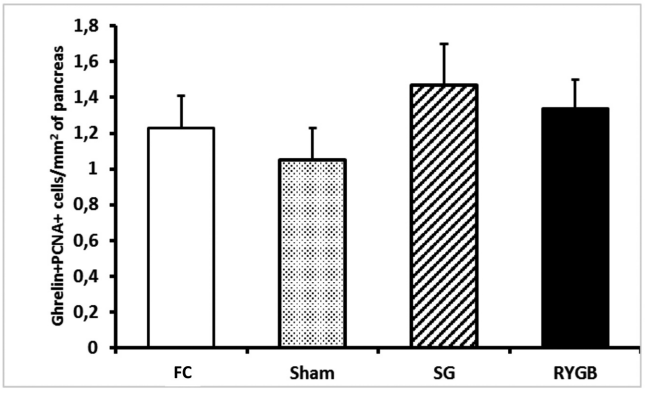

Fig. 5. Proliferating -ghrelin positive cells presented as PCNA/ghrelin positive cells/mm^2 of pancreas. Fasting control (FC, white bar), Sham (dotted bar), SG (striped bar) and RYGB (black bar) groups are presented in the X axis. Values are represented as mean ±EEM (*p<0.05) (**p<0.01). Data obtained were FC: 1.23±0.18; Sham: 1.05±0.18; SG: 1.47±0.23; RYGB: 1.34±0.16.

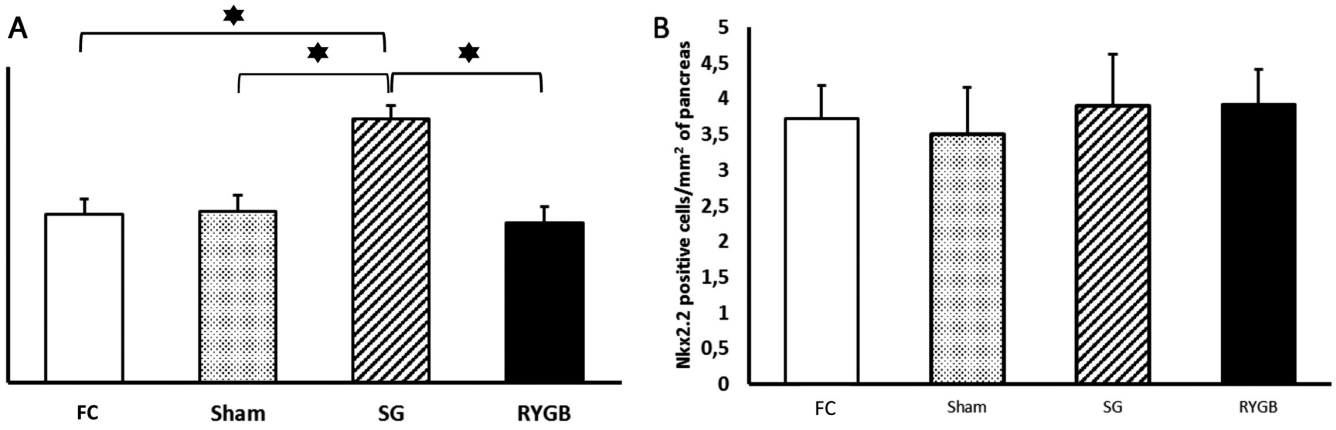

Fig. 6. A. Pancreatic endocrine progenitor cells presented as NGN3 positive cells/mm^2 of pancreas. Fasting control (FC, white bar), Sham (dotted bar), SG (striped bar) and RYGB (black bar) groups were presented in the X axis. Values are represented as mean ±EEM (*p<0.05). Data obtained are FC: 4.79±0.43; Sham: 4.87±0.46; SG: 7.5±0.4; RYGB: 4.55±0.46. **B.** Nkx2.2 positive cells number. Values are represented as mean ±EEM. in CA: 3.72±0.47; Sham: 3.5±0.66; SG: 3.9±0.73; RYGB: 3.92±0.49.

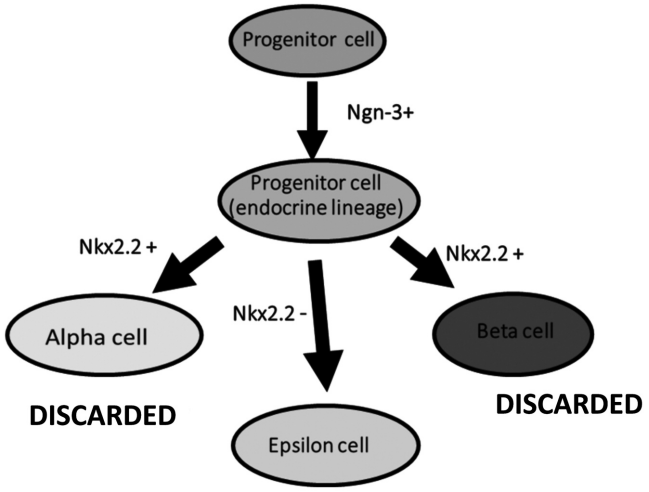

Fig. 7. a. Diagram of pancreatic endocrine cell differentiation. Neurogenine-3 (NGN3) transcription factor is expressed in endocrine progenitor cells. Nkx2.2 is another transcription factor expressed in the differentiation from endocrine progenitor to β or α cells, but not to ε-cells. **B.** Nkx2.2 absence discard β or α cells as final destination of endocrine progenitor cell differentiation.

assay the appearance of new ε-cells from endocrine progenitor cells, pancreas samples from all the groups studied were immunocytochemically linked with anti-NGN3 antibodies. Neurogenin-3 (NGN3) is a transcription factor used as a pancreatic endocrine progenitor marker. A high number of endocrine progenitor cells was found in rats after SG (p<0,05) versus the three other groups -even RYGB- (Fig. 6A).

Finally, Nkx2.2 cell differentiation marker was employed to study the specific ε-cell differentiation path. A low number of Nkx2.2 positive cells were found in the four studied groups (Fig. 6B). On the contrary to neogenesis assay, in the SG group no significant differences were found versus the other three groups.

Discussion

We report an approach to pancreatic release of ghrelin after Sleeve Gastrectomy (SG) and Roux en-Y Gastric Bypass (RYGB) in experimental models, developed in non-obese, normoglycemic rats. Ghrelin is a well-known hormone secreted mainly in stomach mucosae. Reports have shown a 35-45% reduction of ghrelin blood levels after total gastrectomy in humans. Although some studies did not find the same reduction, it was related to the plasma circulating deacylated/acylated isoforms (Ezquerro et al., 2019; Mani et al., 2019). This coherent consequence is related since to the broad stomach portion resected in these surgeries (Ariyasu et al., 2001; Date et al., 2002). These concepts lead us to think about the alternative source of ghrelin. In our study, the immunohistochemical analysis showed a significant increase of ε-cell in pancreas after SG, but not after RYGB (Fig 3). This was preceded by an initial depletion of ghrelin basal serum levels after SG, compared to the permanent reduction of fasting blood ghrelin levels in RYGB rats (Fig 4). Meanwhile, ghrelin serum levels were maintained in control groups. These findings suggested a pancreatic histophysiological response to the resection of gastric ghrelin-producing cells in SG.

In addition, the presence of a residual ghrelin-producing cell population in pancreas after birth in humans and rodents was reported (Ariyasu et al., 2001; Popovic et al., 2005; Andralojc et al., 2009). Otherwise, some endocrine cell populations, such as beta cells, keep

the capability to respond to certain injuries or biological circumstances (as pregnancy) with a remarkable expansion (Zhu et al., 2017). These concepts supported our attention to the pancreas as the replacing source of ghrelin after bariatric surgeries. However, other ghrelin-releasing tissues exist (such as small intestine, testis, brain, cerebellum, pituitary, lung, skeletal muscle or salivary gland) which could be responsible for the blood ghrelin level elevation after SG (Ghelardoni et al., 2006). However, according to our results, we did not find increasing ghrelin levels after RYGB during the studied period (Fig. 4). Thus, after this surgery, other ghrelin-releasing sources did not appear to be enhanced. In other words, our data about ghrelin basal levels supported a recovery after SG from day twenty-one to the end of the experiment. It suggested a possible expansion of residual pancreas ε-cell population as being responsible for circulating ghrelin.

Assuming the pancreatic ε-cell population expansion, we analyzed the cellular mechanisms involved. As figure 5 shows, we probed cellular proliferation, by using the PCNA marker, as the mechanism to increase the ghrelin-producing cells in the pancreas. No significant differences were found between any of the studied groups. This result was expected attending to previous studies in Wistar rats, which showed no differences in β-cell proliferation between SG and control groups (Camacho-Ramírez et al., 2017).

The capability of β-cells or α-cells to co-release ghrelin could explain the elevation of ghrelin basal level in SG rats after surgery (Heller et al., 2005). However, this mechanism does not explain the increased number of ghrelin positive cells (Fig. 3). Moreover, in this argument, we have not found double staining ghrelin/insulin or ghrelin/glucagon positive cells.

Other explanations than pancreatic ε-cell expansion could be the appearance of new ε-cells due to the neogenesis of ductal stem cells. To stain this cellular mechanism, we employed the NGN3 marker, a well-known endocrine progenitor cell-lineage marker (De Groef et al., 2015). Figure 5 shows a significant number of NGN3 positive cells in SG group, but not in controls or RYGB rat pancreas. This suggests an increase of stem cell differentiation of pancreatic endocrine cells after SG.

However, NGN3 positive cells can come from β-cells or α-cells too. Thus, we employed other endocrine lineage cell marker -NKx2.2-. NKx2.2 is present in the differentiation route to β-cells and α-cells from endocrine progenitors but not in the differentiation to epsilon cells (Fig. 6) (Sakata et al., 2019). No NKx2.2 positive cell was found in any of the studied groups. This data identified the ε-cells as the final cell of the differentiation process.

All the data explained the ε-cells expansion after SG by a differentiation mechanism. Nevertheless, other phenomena could be involved, such as a decrease of ε-cell apoptosis after SG. However, it did not seem to be a powerful enough mechanism to explain ε-cell expansion three months after surgery. Supporting this idea, no-single change in cell-apoptosis ratio was found in other pancreatic endocrine cells in the same models (Camacho-Ramírez et al., 2017).

We conclude that pancreatic ε-cell expansion is a physiological response to a severe injury in the main source of ghrelin. After sleeve gastrectomy, the number of ε-cells was increased mediated by stem cell differentiation, but not after RYGB. This different behavior may be related to the different amount of remnant ghrelin-producing mucosae in the stomach after every surgery (Pereferrer et al., 2008). It is possible that the low initial serum ghrelin level after SG triggers the differentiation stimulus in pancreas. But however ghrelin has not been proposed as a stem or mesenchymal cell differentiation promoter (Ye et al., 2018; Fan et al., 2019). Thus, another signaling pathway must be involved in this feedback.

All these findings let us draw an image of endocrine pancreas twelve weeks after surgery with significant results. We cannot determine the possible changes in longer survival periods. We did not discard other possible changes in peripheral ghrelin-producing tissues after SG or RYGB. However, we conclude the importance of pancreas in ghrelin releasing in some specific situations, such as the sleeve gastrectomy.

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Authorship statement. The authors declare that all of them have participated actively in one or more steps related with this work, from the intellectual design, the surgical procedures, the laboratory techniques, to the drafting of the manuscript.

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