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A surgical model of short bowel syndrome induces a long-lasting increase in pancreatic beta-cell mass

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Summary. Several surgical techniques are used nowadays as a severe treatment for obesity and diabetes mellitus type 2. These techniques are aggressive due to drastic changes in the nutrient flow and non-reversible modifications on the digestive tube. In this paper we present the effects of a massive intestinal resection on the pancreas. Results have shown that short bowel technique is less aggressive to normal anatomy and physiology of the intestinal tract than Gastric bypass or biliopancreatic diversion (e.g.). In this paper we reproduce a model of short bowel syndrome (SIC), with similar surgical conditions and clinical complications as seen in human cases. This work was conducted on normal Wistar rats, with no other concurrent factors, in order to determine the effects on normal pancreas islets. We measured pancreatic implications by histo-morphometric studies, which included beta-cell mass by immunocytochemistry, and apoptosis/proliferation test with TUNEL technique and Ki-67. Briefly, we reported on an increased relative area of the islets of the pancreas, as well as an increase in the average size of islets in the SIC versus the control group. Furthermore we stated that this increase in size of the pancreatic islets is due to the mechanisms of proliferation of beta cells in animals undergoing SIC. These goals could reveal a direct influence of surgical modification of the digestive tract over the pancreatic beta cell homeostasis. In this sense, there are many potential stimulators of intestinal adaptation, including peptide hormones and growth components which are associated or involved as effectors of the endocrine pancreas.

Key words: Pancreas, Diabetes, Bariatric-surgery, Insulin-Secreting Cells, Short-bowel syndrome

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Introduction

Obesity has become a major public health problem due to its prevalence, costs and negative effects on health. The World Health Organization predicts that in the year 2015 obesity, as defined by a body mass index (BMI) of at least 30 kg/m^2, will affect approximately 700 million people around the world.

Type 2 diabetes mellitus (T2DM), characterized by insulin resistance and relative insulin insufficiency can be considered one of the most important obesity-associated pathologies. It is present in more than one-fifth of obese adults and, as reported in a meta-analysis by Buchwald et al. (2004), approximately 20% of gastric bypass candidates suffer type 2 Diabetes. It usually affects people over the age of 40, but it may also occur earlier, especially in populations with high diabetes prevalence. The prevalence of T2DM is currently estimated in 5-10% of the population with an increasing global trend, especially in children and adolescents (Wild et al., 2004).

Bariatric surgery was first used in 1954 to induce weight loss by the surgeons Kremen et al., based on their observations in short bowel syndrome (SIC) patients and other pathologies in which bowel length or gastric capacity are surgically reduced. The same results were

seen in experimental studies using animals since the end of XIX century and XX century (Brenizer, 1929; Booth, 1959), and similar considerations were reported in human studies (West et al., 1939; Meyer, 1946; Buchwald, 1970; Casey and Martin, 1981).

Currently it represents the most important therapeutic alternative to induce weight loss in cases of morbid obesity. Obese patients suffering from T2DM, who undergo bariatric surgery, experience diabetes remission in a significant percentage of cases. Surprisingly, diabetes remission happens before weight loss, suggesting a relationship between digestive tract modification and changes in glucose homeostasis. The incidence of diabetes remission varies according to the surgical technique applied. In the meta-analysis by Buchwald et al. (2009), including a total of 135,246 patients studied for 16 years, found diabetes remission in 56.7% of patients who underwent laparoscopic adjustable gastric banding, 80.3% of patients who underwent gastric bypass, 79.7% of patients who underwent gastroplasty and 95.1% of patients who underwent biliopancreatic diversion/duodenal switch.

Restrictive and malabsortive components are normally present in bariatric surgery procedures and both of them are considered responsible for the anti-diabetogenic effects. The mechanisms responsible for these remain unknown. Theories to explain these anti-diabetes effects may be classified in three different groups for academic purposes: the Ghrelin hypothesis postulates that compromised secretion of the hormone Ghrelin from the stomach might be related to the glucose homeostasis improving effects of some types of bariatric surgeries (Cummings and Shannon, 2003; Holdstock et al., 2003). The lower intestinal hypothesis proposes an accentuated GLP-1 secretion due to expedited delivery of unabsorbed nutrients in the distal gut as anti-diabetes stimulus (Koopsmans et al., 1984; Bose et al., 2009; Eickhoff et al., 2014). The upper Intestinal hypothesis suggests that preventing the contact of ingested nutrients with a segment of the proximal small intestine, mainly the duodenum, exert a direct anti diabetic effect via unidentified mechanisms (Rubino and Marescaux, 2004; Duan et al., 2014; Salinari et al., 2014). However, none of these theories alone can totally explain the observed improvement in glucose homeostasis attained with both restrictive and malabsortive procedures, although an altered secretion of gastrointestinal (GI) hormones may be the basis of these effects.

Surgical modification of the GI tract affects the secretion of different GI hormones and peptides such as Ghrelin and GLP-1. It is well known that these hormones have the capacity to induce proliferation of beta cells (Drucker, 2003; Kerem et al, 2009; Doss and Smith, 2012). However, very little is known about the effects of bariatric surgery on pancreatic beta cell turnover or even insulin resistance (Salinari et al., 2014).

In our work, we aimed to investigate the potential effects of a purely malabsortive technique with preserved duodenum in pancreatic beta cell proliferation. We performed a surgical reduction of 50% of the central portion of the small intestine in a set of non-obese Wistar rats to avoid interference with presurgical insulin resistance (Collantes-Perez et al, 2004) and evaluated its effects on beta cell proliferation/apoptosis balance. An increase of pancreatic islet relative area and beta cell proliferation was observed in intervened animals, revealing a direct effect of digestive tract alteration on pancreatic beta cell homeostasis.

Materials and methods

Animals

All animal procedures were performed with the approval of the Cádiz University School of Medicine (Cádiz, Spain) Committee for the Ethical Use and Care of Experimental Animals. Wistar rats were kept under conventional conditions in an environment-controlled room (20-21°C, 12h light-dark cycle) with water and standard laboratory rat chow available *ad libitum*.

Surgical procedures

Young adult male Wistar rats (n=12) of approximately 250 g, were subjected to a presurgical protocol consisting of a 24 hours fasting period. They were then anaesthetized with isofluorane (Isoflo, Abbott 571329.8) and a surgical resection of 50% of their small intestine was performed by laparotomy. Briefly, after abdominal midline incision, we identified the Treitz's angle and the ileocecal valve as anatomical references. The bowel between these points was exposed and measured. We then made a resection of the central 50%, followed by an end-to-end anastomosis with 5-0 monoplane silk suture, leaving the proximal half of the jejunum and the distal half of the ileum intact (Fig. 1). Short bowel syndrome group consisted of 6 animals. Sham group underwent the laparotomy and bowel section and anastomosis without resection (n=6). Overnight, post-operatory rats only received water. For the next five months animals were fed with regular rat chow and their weight was monitored every two weeks.

Tissue preparation and Immunohistochemistry

Animals were sacrificed five months after surgery by an intraperitoneal Chloral Hydrate overdose to avoid unnecessary pain. Pancreas were immediately removed and fixed in Bouin's solution overnight at 4°C, followed by a 72 hours postfix in Formalin at room temperature (Pearse, 1968). They were then rehydrated using graded alcohols and xylol and embedded in paraffin. Serial 10 μm-microtome sections were obtained, mounted on poly-lysinated slides, and stored until processed.

Sections were deparaffinized in xylol and rehydrated in degraded alcohol series and distilled water. Endogenous peroxidase activity was inhibited with 3% H_2O_2 solution treatment for 30 min at room temperature.

Sections were then washed twice in phosphate buffer saline (PBS, pH 7.4).

To perform histomorphometric studies, insulin was stained using a mouse anti-insulin monoclonal antibody (Sigma, I-2018), a peroxidase conjugated goat anti mouse IgG antibody plus streptavidin complex (Sigma, Mouse Extra-2). Samples were revealed with a solution of 0.3 mg/ml of 3,3'Diaminobenzidine (Sigma, D5905) in the presence of 0.2 μl/ml of H_2O_2 under microscopic control. To determine beta-cell area, the insulin-positive areas were measured using a microscope equipped with a digital camera and the image analysis Cell D software (Olympus, Hamburg, Germany). The investigators who performed the measurements did not know which experimental group the samples belonged to. Beta-Cell area was expressed as the insulin-positive area/total pancreatic area ratio.

Detection of proliferation and apoptosis

Proliferation was assessed by double immunostaining using polyclonal rabbit anti-Ki67 (ABIN1582250) and monoclonal mouse anti-insulin (Sigma, I-2018) antibodies. After deparaffination and rehydration, pancreatic sections were incubated for 30 min with 0.2% Triton x-100 in PBS containing 3% BSA to permeabilize the tissue, and incubated overnight at 4°C with anti-Ki67 and anti-insulin antibodies. Stained sections were revealed using anti-rabbit IgG antibody (Alexa-488 conjugated) and anti-mouse IgG (Alexa-546 conjugated) antibodies (Molecular Probes, Inc., Eugene, OR).

Beta-Cell apoptosis was determined using the Dead-End Fluorometric-terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine-5'-triphosphate nick end labelling (TUNEL) System (Promega, Madison,WI) according to the manufacturer's instructions, and simultaneously stained using monoclonal mouse anti-insulin (Sigma-Aldrich), revealed with an anti-mouse IgG (Alexa-546 conjugated) antibody (Molecular Probes Inc; Eugene, OR, USA).

To determine the proliferating or apoptotic fraction, insulin+/Ki67+ cells or insulin+/TUNEL+ cells and islets area were quantified in a total of 25 islets per condition. Slides were analyzed using a fluorescence microscope equipped with a digital camera and the image analysis Cell-D software (Olympus, Hamburg, Germany). The results were expressed as number of insulin+ /Ki67+ cells/mm^2 of islet or insulin+ /TUNEL+ cells/mm^2 of islet.

Statistical analyses

Results are presented as means±SEM of measurements performed in at least five animals per group. Statistical comparisons were performed either by Mann-Whitney' U test or by ANOVA followed by Sheffe's test. All p values less than or equal to 0.05 were considered statistically significant.

Results

Effect of surgery in weight gain

To evaluate the effect of small bowel resection in animal weight gain, this parameter was quantified every

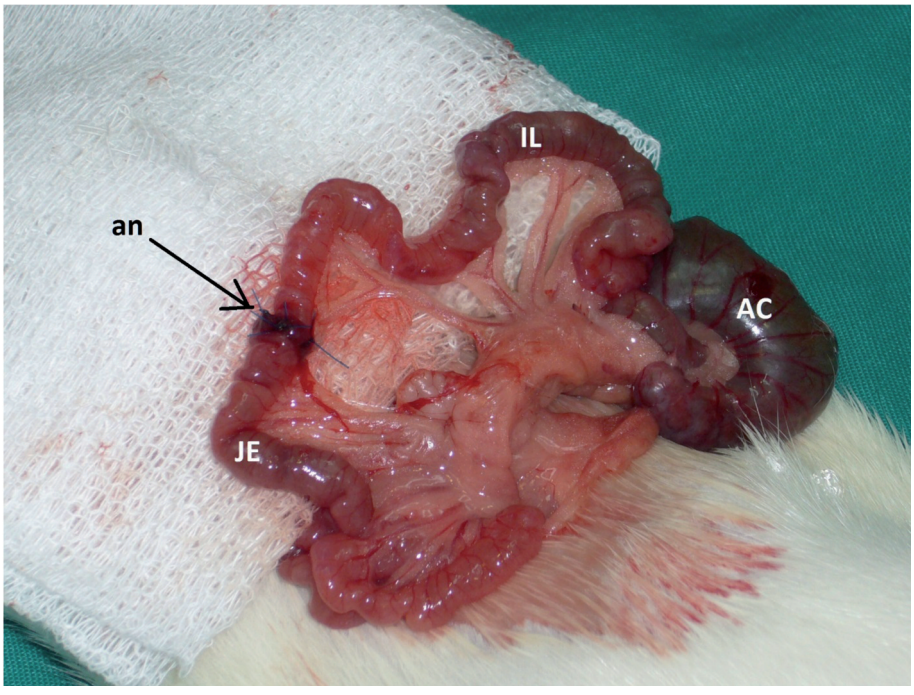

Fig. 1. Remnant small intestine after surgical procedure. The suture (an: anastomosis) is clearly seen at the junction between jejunum (JE) and ileum (IL), marked with an arrow. AC marks the ascending colon.

two weeks, for five months after surgery. As explained in Fig. 2, no statistically differences were observed between sham surgery and intestinal resection in weight gain, although animals that suffered the SIC showed a smaller increase in the normal rate of weight gain compared to controls.

Changes induced by small bowel resection in beta cell population

Insulin positive pancreatic area/total pancreatic area ratio was evaluated five months after surgery in paraffin embedded pancreatic tissue sections immunostained using a mouse anti-insulin antibody (Fig. 3). A significant enhancement in insulin positive pancreatic area/total pancreatic area ratio was observed in the experimental group with intestinal resection compared with Sham rats (Fig. 4A).

In addition, we analyzed total number of islets/total pancreatic area (mm^2) and an increment in this ratio in the group of rats that underwent intestinal resection was also observed five months after surgery (Fig. 4B). With these figures we described that the islets appeared larger in the SIC against the sham group, and the number of islets was increased, although this fact was not statistically significant.

Effect of small bowel resection in beta cell turnover

To evaluate the role of beta cell proliferation in relative islet area increment observed after surgery, the percentage of proliferating insulin-positive cells was quantified. We used paraffin embedded pancreatic tissue sections taken from rats with intestinal resection and sham surgery (Fig. 5). These proliferative beta cells were stained with anti-insulin/ki67 antibodies. An increase in ki67 positive/insulin positive cell numbers was observed in rats with intestinal resection in relation to control animals five months after the intestinal resection (Fig. 6).

Changes in beta cell apoptosis ratio could also contribute to these modifications in islet relative area. For this reason, TUNEL technique and insulin staining were simultaneously used to evaluate the beta cell apoptosis ratio (Fig. 7). No significant differences were found in beta cell apoptosis rate between experimental groups five months after intestinal resection (Fig. 8).

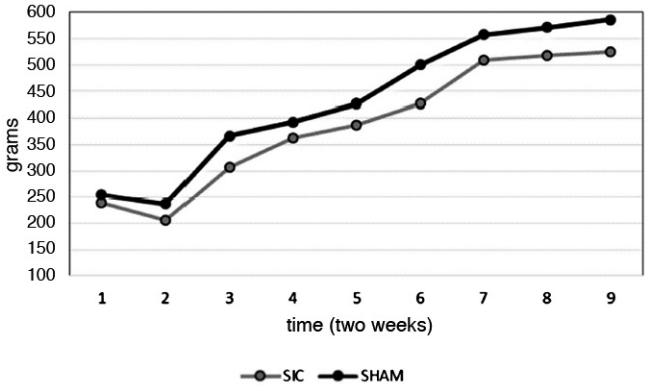

Fig. 2. Animal weight was quantified every two weeks and weight gain (in grams) (Y axis) is represented in both experimental groups along time (X axis) as mean ± SEM. The figure showed a pattern of weight gain starting 4 weeks after surgery. Point 1 of the X-axis represents the weights of the rats at surgery time. In the first two weeks after surgery there was no increase in weight. Both experimental groups showed a decrease in weight in these weeks, due to surgical stress (point 2 of the X axis). No-significant difference was observed between both groups. The figure shows a delay between the last point and sacrifice, due to the experimental procedure.

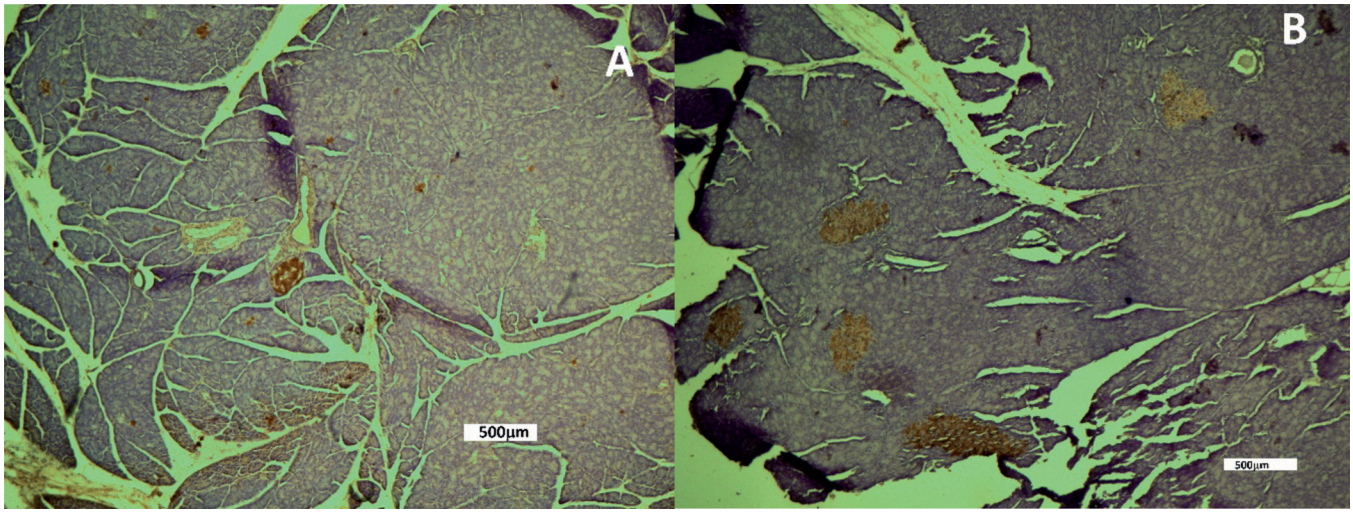

Fig. 3. Insulin-positive immunostained islets, counterstained with haematoxylin. **A.** Control sample. **B.** SIC sample. Larger islets were observed in the short bowel syndrome.

Changes induced by surgery in pancreatic islet size distribution

The effect of surgery on the size of pancreatic islets distribution was evaluated in paraffin embedded tissue sections of pancreas immunostained with mouse anti-insulin antibody. We observed a significant enhancement in the number of large pancreatic islets (with areas >5000 μm^2) in resected rats compared with Sham rats. No different values were observed in other ranks of islet size between both groups (Fig. 9).

These data reaffirmed that observed in the other figures and results, in the sense that the main mechanism related to beta mass expansion in SIC must be proliferation rather than neogenesis. Proliferation of pre-existing beta cells increased the area of islets, but not the number of islets (Fig. 4), and for this reason the immunohistological analysis showed an increase of large islets in the SIC (Fig. 9). Instead, the number of small islets in the pancreas would be related through a mechanism of transdifferentiation of the epithelial ducts or stem cells.

Discussion

The positive direct effects of bariatric surgery techniques on the clinical T2DM course are well known and there are a set of hypothesis to explain them. One of the most important hypothesis presents duodenal exclusion as a major inductor of changes that result in glucose homeostasis improvement. These effects take place short a time after surgical intervention (Lautz et al., 2011; Schauer et al., 2014). In this study we used an experimental surgery in which the entire duodenum is

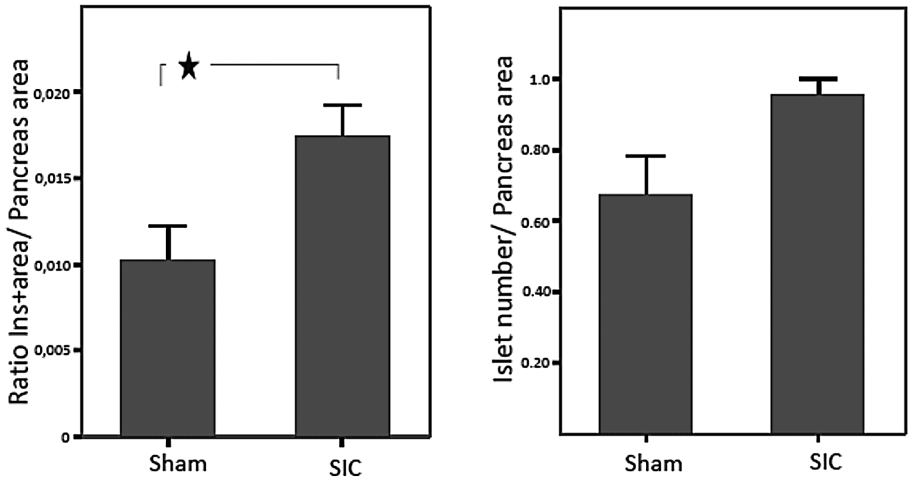

Fig. 4. A. Insulin positive pancreatic area/total pancreatic area ratio is presented in bar graph as means ± SEM. *P≤0.05. **B.** Number of pancreatic islets in relation to pancreatic area was determined and represented as mean values ± SEM. Thus, in these figures we reported that the islets appeared larger in the short bowel syndrome against the sham group. There was an increase in number of islets, but these data were not statistically significant. These data were consequent with cellular proliferation of preexisting beta cells. A neogenesis process in the SIC would be related with an increased in the number of islets, generated from pancreatic ducts.

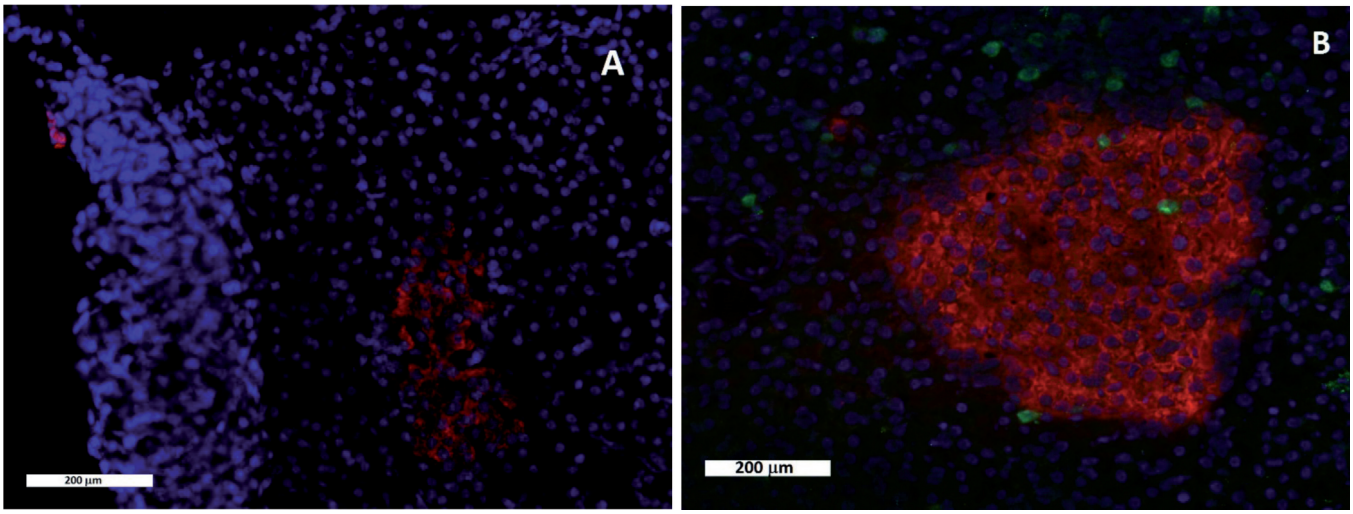

Fig. 5. Proliferating insulin positive cells stained with anti-Ki67 antibody. **A.** Control sample. **B.** SIC sample. The number of insulin positive cells, which express the protein Ki67 (green-fluorescent Alexa Fluor 488) related to proliferation process was increased in the SIC rats.

preserved as well as the proximal half of the jejunum and distal half of the ileum, representing a resection of approximately 50% of the total length of the intestine (Collantes-Pérez et al., 2004). We performed this surgery on normal weight Wistar rats, because if we used an animal model of obesity, insulin resistance or even diabetes, the effects on glucose homeostasis and pancreatic beta cell apoptosis/proliferation balance could have been influenced by the presurgical condition. By using normal healthy weight animals, the changes observed can only be interpreted as a direct effect of the surgery itself.

Various diseases require a surgical resection of a variable length of the small intestine in humans, and their nutritional consequences have been well studied. Necrotizing enterocolitis and intestinal atresia in children, as well as vascular thrombosis, malignancies and Crohn's disease in adults are the most frequent indications of intestinal resection. Our surgical experience in these cases has demonstrated that resections of up to 50% may be well tolerated without the need for nutritional support. Our data on animal weight monitoring during the study is in agreement with this observation in humans, since no surgery-induced changes in weight gain were found (Fig. 2).

We examined the histomorphometric features of pancreatic islets five months after surgery, comparing sham and 50% small intestine resected animals. The experimental group with the bowel resection showed a significant increment of relative area of pancreatic islets and islet number (Fig. 4A,B). Both figures showed that the islets appeared larger in the SIC against the Sham group, but there was no increase in the number of islets in either group. These data were related to the pancreas total area. These findings could be the consequence of a cellular mechanism of proliferation, where beta cells contribute homogeneously to the islets area. The proliferation increases the beta cell mass from the preexisting beta cells (Yesil and Lammert, 2008). Meanwhile, the neogenesis process, which would be related to an increase in the number of small islets generated from stem cells or epithelial cells located in the pancreatic ducts, is not coincident with our observations.

To clarify the mechanism underlying the observed islet area increment, pancreatic beta cell proliferation and apoptosis were quantified. No surgery induced changes were observed in beta cell apoptosis rate (Fig.

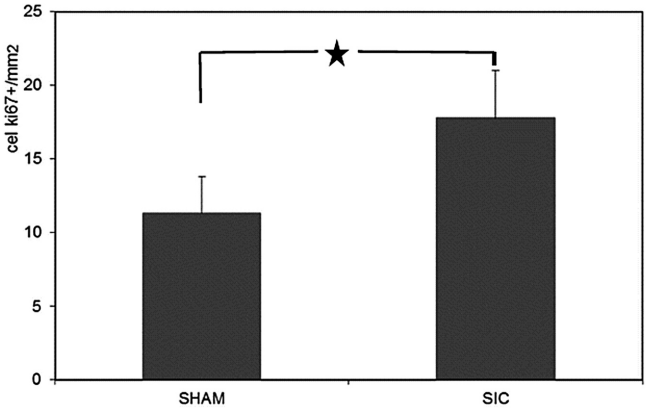

Fig 6. Proliferating beta cells were quantified in pancreas sections from rats with intestinal resection and sham five months after surgery. Beta cell proliferation rate is shown in bar graph as means ±SEM, where the Y axis represents the number of Ki 67 positive/insulin positive cells per islet area expressed in mm2. *P<0.05. Thus, these data show that in the bowel resection the number of proliferating beta cells increased compared to surgical controls. This data was statistically significant.

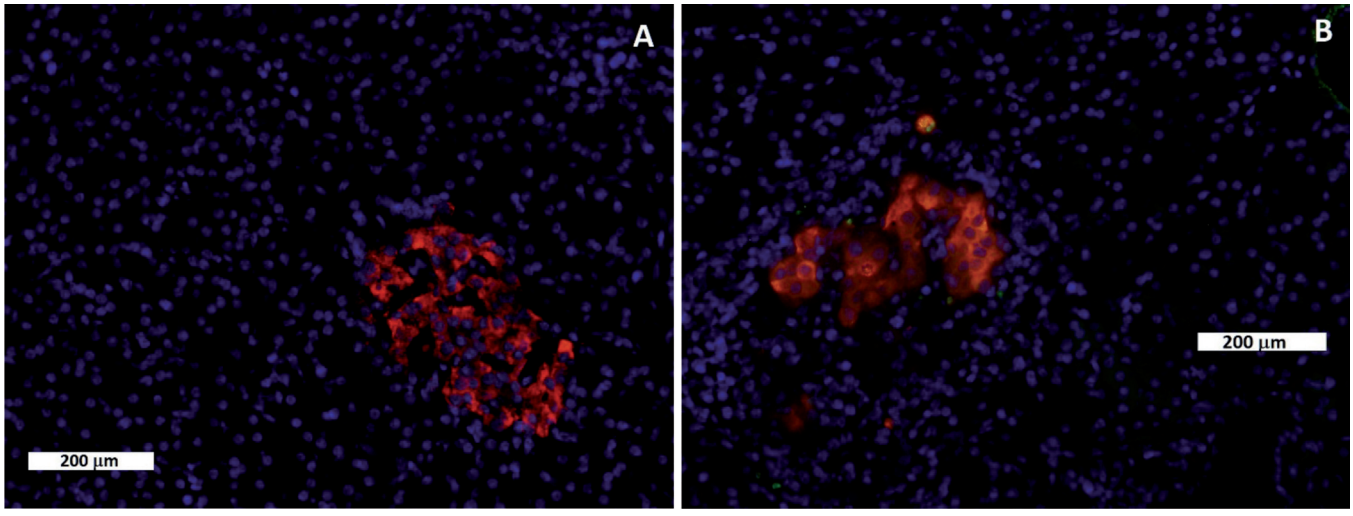

Fig. 7. TUNEL technique. **A.** Control sample. **B.** Some isolated positive cells (green-fluorescent Alexa 488) in a SIC sample. Insulin positive cells stained in islet with red-fluorescent (Alexa Fluor 546).

8). On the other hand, beta-cell proliferation rate was significantly higher in the experimental animals that underwent the surgical resection (Fig. 6). The sum of these events could explain the increment of islet area and number. An increase of total pancreatic islet area in response to different stimulus is due to both neogenesis and beta cell replication processes (Yesil and Lammert, 2008; Bonner-Weir et al., 2010). An indirect measurement of the participation of neogenesis and beta cell replication in islet area increment is the study of islet size distribution. An increment in beta cell clusters and/or small islets indicates a major participation of neogenesis in islet area increment, while an increment of large size islets suggests an increase in beta cell replication as the main mechanism responsible for the observed islet area increment. In our study, a 50% of small intestine resection preserving duodenum, proximal jejunum and distal ileum, induced a long term increment of large size islet number (Fig. 9). As stated before, this observation suggests that beta cell replication is the main mechanism implicated in surgery induced islet area increment.

Some patients suffer severe hypoglycemic episodes after bariatric surgery for morbid obesity. To control these episodes, patients often require partial pancreatectomy. Pathological studies of the resected pancreatic portions show typical images of nesidioblastosis (Service et al., 2005). Although no nesidioblastosis images were observed in our study group, we found an increased islet area and number, which could suggest a different stage of a common process. Recently, the effects of intestine alteration in rodent beta cell functionality have been studied using different surgical models. Isolated islets from duodenal-jejunal bypass performed on Western diet obese Wistar rats have shown an enhancement in beta cell glucose responsiveness (Araujo et al., 2012). This is in accordance with data from GK rats subjected to duodenal-jejunal exclusion, in which pancreatic islet insulin mRNA expression was higher than in controls (Donglei et al., 2012). Another feature supporting the duodenal-jejunal bypass induced improvement in glucose homeostasis in GK has been recently reported by Speck et al. (2011). They have reported an increment of beta cell area and a reduction of islet fibrosis induced by surgery. Similar results have been obtained in rodents and humans after Roux–en-Y gastric bypass (Li et al.. 2010; Rabiee et al., 2011; Yu et al., 2013). A common feature among these reports was duodenal exclusion, coherent with the upper intestinal hypothesis. The main problem with duodenal exclusion to the transit of nutrients is the adverse effect on the absorption of

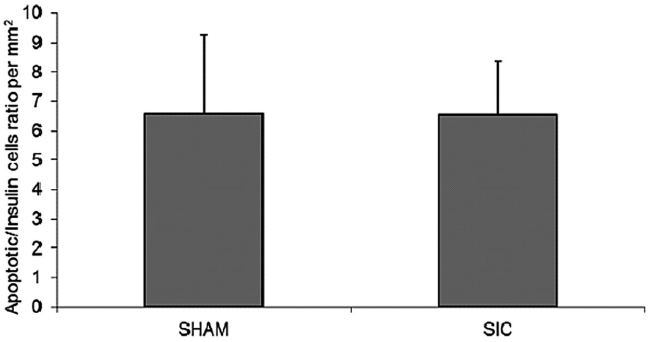

Fig. 8. Apoptotic beta cells were quantified in pancreatic sections from rats with intestinal resection and sham five months after surgery. Apoptotic beta cell ratio is presented in bar graph as means ±SEM of TUNEL positive/insulin positive cells per islet area expressed in mm^2. There were not differences between groups.

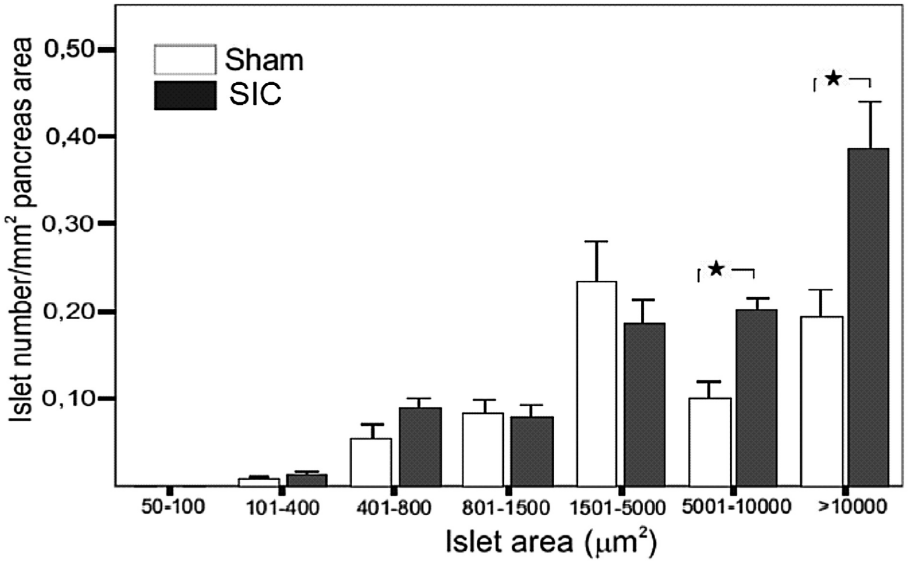

Fig. 9. Size distribution of islets was calculated in pancreatic sections from rats with intestinal resection and sham five months after surgery, by quantifying islet number belonging to each area value interval and is represented in bar graph as means ± SEM of islet number. *P<0.05. These data showed that the islets not only were increased in number in SIC, but they were larger than in surgical control. When we grouped the islets by the size of the area, the graph showed that in the Sham group the islets used to be between a wide ranges of 2000-10000 μm^2. Meanwhile, in SIC an abundant number of islets presented a large area superior to 0.01 mm^2

different micronutrients which may have negative long term consequences. Instead of this, in our surgical model the duodenal segment is preserved. Furthermore the nutritional status is maintained without other undesirable effects such as chronic diarrhea, dumping syndrome, etc. In conclusion, using an animal model in which overweight is not a participating variable, our work demonstrates the effect of a non-weight-loss-inducing intestinal surgery in beta cell increase rate. This effect is mediated by duodenal exclusion. Our data suggest that other different mechanisms than those involved in the proximal gut hypothesis should be implicated in the events of turnover and expansion of adult beta cell mass. Moreover, the data suggest that the use of this surgical technique with low effects on nutrition can be a good approach to physiopathological mechanism in T2DM, mainly in patients in whom obesity is not an associated problem.

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