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Organic consequences of ileal transposition in rats with diet-induced obesity.

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ABSTRACT

INTRODUCTION: The clinical management of metabolic syndrome - especially diabetes mellitus type 2 - is notoriously complex due to the progressive nature of this disease. At present, there is a need for a surgical procedure that is effective for the treatment of diabetes mellitus type 2, even in non-obese individuals. The isolated ileal transposition theory could lead to an effective alternative therapy. This intervention has not yet been performed in humans, and there are no reports of its use in an experimental model of diet-induced metabolic syndrome.

OBJECTIVES: The objective of this study is to evaluate the physiological effects of ileal transposition in rats with diet-induced metabolic syndrome. The effects of this procedure on glucose and lipid metabolism will be assessed.

METHODS: Forty 12-week-old male rats (albino *Rattus norvegicus*, Wistar, 2BAW, heterogeneous) will be divided into four groups of 10 animals each: the ileal transposition group (TG) comprising animals on a hypercaloric-hyperlipidic diet; the sham group (SG) containing animals that receive the same diet and undergo the sham surgery; control group 1 (CG1), which will receive a hypercaloric-hyperlipidic diet and will not undergo surgery; and control group 2 (CG2), which will consume standard feed and will not undergo surgery. The surgeries will be performed in 20-week-old animals. Blood samples for laboratory testing will be collected from 12-week-old animals on the day of surgery and after eight postoperative weeks, following determination of the weights of the animals and the administration of anesthesia. The levels of serum glucose, insulin, triglycerides, total cholesterol and fractions, glucagon-like peptide-1, C-peptide and glycated hemoglobin will be assessed in all of the animals. The insulin tolerance test will be performed using PRISMA software, and insulin resistance will be calculated by the HOMA-IR indirect test. On specific days, two 20-week-old rats will be separated and randomly distributed in TG and SG. These animals will be followed until the eighth postoperative week. Subsequently, they will be euthanized, and the retroperitoneal and periepididymal fat deposits will be collected and weighed using a precision scale. In addition, the pancreas, liver and intestinal segments will be sent for pathological and immunohistochemical studies.

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1. INTRODUCTION

In recent decades, the world's population has experienced a significant increase in the prevalence of obesity.[1] At the transition of the millennium, approximately two thirds of adults in developed countries were obese, and nearly 5% of them suffered from morbid obesity.[2] Morbid obesity predisposes patients to comorbidities that affect almost every organic system,[3] and the association of obesity with insulin resistance (IR), systemic hypertension (HBP), dyslipidemia and diabetes mellitus type 2 (DM2) results in metabolic syndrome (MS), which represents an important risk factor for the development of cardiovascular disease.[4] In 1998, the World Health Organization (WHO) established that MS is characterized by the association of IR or DM2 with two or more of the following criteria: (1) a blood pressure (BP) greater than or equal to 160/90 mmHg, (2) hyperlipidemia, defined as triglyceride levels above 150 mg/dL and/or HDL cholesterol below 35 mg/dL in men and below 39 mg/dL in women, (3) central obesity with a waist/hip ratio of greater than 0.9 in men and greater than 0.85 in women and/or a BMI greater than 30 kg/m^2 (4) and microalbuminuria of at least 20 mg/min or an albumin/creatinine ratio greater than 20 mg/g.[4] Furthermore, these individuals often present cardiovascular disease, hypoventilation syndrome, asthma, sleep apnea, stroke, pseudotumor cerebri, arthropathies, several types of cancer, urinary incontinence, cholecystolithiasis, gastroesophageal reflux disease and depression.[4-6]

Obesity results in a shortened life span.[7] This reduction is approximately 12 years in the morbidly obese in comparison to individuals with a normal weight and is directly proportional to increases in the body mass index (BMI).[8] In the near future, morbid obesity will replace smoking as the leading cause of death in developed countries.[9] Currently, in the United States alone, there are more than nine million morbidly obese patients who require assistance.[2] However, the conservative treatment of morbid obesity with changes in diet and exercise, lifestyle modifications and medication only very rarely result in adequate levels of weight loss that are maintained.[10] In fact, four long-term studies investigating the conservative treatment of obesity demonstrated a mean weight loss of only 4%. A prospective controlled study showed that conservative treatment over ten years resulted in an increased body weight of approximately 1.6% compared to a weight loss of 13.2% with the adjustable gastric band and 35% with gastroplasty reduction and gastrojejunal derivation.[15]

Since the advent of minimally invasive surgery, there has been a considerable increase in gastrointestinal procedures that result in significant and lasting weight losses with low complication rates.[16-18] Unlike the conservative approach,[19] bariatric surgery determines the improvement or resolution of obesity-

associated diseases in 70% to 100% of patients.[18] In addition, the mortality rates in morbidly obese patients are significantly reduced following bariatric surgery in comparison to those determined in groups of obese patients undergoing medical treatment.[20,21] Therefore, bariatric surgery is clearly the most effective treatment for morbid obesity; it promotes a significant and sustained weight loss, improves or resolves associated comorbidities and prolongs the life expectancy of patients.[22]

The gold standard surgical treatment[23] for the treatment of morbid obesity is the vertical banded gastroplasty with the gastrojejunal shunt using the Roux-en-Y technique (Fobi-Capella modified surgery[24,25]) because it achieves the goals of lasting weight loss and the control of comorbidities.[16] The weight loss is usually attributed to a decline in the gastric pouch volume together with intestinal malabsorption. In turn, the control of comorbidities after Roux-en-Y gastroduodenal bypass - especially those resulting from glucose metabolism disorders - are generally attributed to an actual decrease in body mass. Nevertheless, endocrine effects potentially control blood glucose and promote early satiety following the Fobi-Capella procedure, even before any significant weight loss.[26]

Additionally, substances that are secreted by the intestine directly into the bloodstream are increased in the peripheral blood following gastroduodenal bypass, such as glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). These proteins can stimulate insulin production by pancreatic beta cells, facilitate the action of this hormone in the transport of glucose into cells and affect the hypothalamus to induce satiety.[27] Moreover, Fobi-Capella surgery drastically reduces the levels of the orexigenic hormone ghrelin, which contributes significantly to the observed reduction of body weight.[28] The levels of ghrelin decrease because the hormone-producing gastric areas (the gastric fundus and the greater curvature) do not receive a food stimulus due to their exclusion from the digestive tract.[28] GLP-1 and the PYY increase in response to the Roux-en-Y gastrojejunal bypass-induced stimulation of L cells, which produce these hormones, and quickly and poorly digested food is observed in the most distal regions of the small intestine.[29] Thus, the reductive vertical banded gastroplasty and the Roux-en-Y gastrojejunal bypass (Fobi-Capella surgery) constitute successful metabolic interventions.

Moreover, the generation of other surgical settings for the digestive tract has been recommended to trigger the appropriate metabolic response to the challenges associated with the surgical treatment of obesity. Thus, diverse surgical techniques are currently being evaluated. Research is also directed toward a surgical cure of comorbidities that are caused by the presence of excess fat. The issue is important because obesity is a condition that demonstrates an increasing incidence and

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prevalence,[30] a universal[31] distribution, no discrimination of age groups and ethnicities and serious consequences to the health of patients due to its association with numerous comorbidities.[32] Obesity is also associated with significant financial implications for the world economy.[33]

Obesity appears to be an important issue among children and adolescents. The growing obesity pandemic has increasingly affected this population, and it can shorten their lives by up to 20 years.[34]

Insulin resistance and diabetes mellitus are conditions that are commonly related to obesity. However, these conditions do not only affect the obese population, because there is an increasing incidence of type 2 diabetes among individuals with a body mass index (BMI) that is in the eutrophic and overweight ranges.[35,36] Insulin resistance is known to be the primary cause of cardiovascular disease in these individuals.[37]

The etiology of insulin resistance in obesity may be due to genetic factors. It has been postulated that natural selection will promote the survival of individuals who carry the "economy" energy gene, as such individuals will be endowed with a greater ability to store calories in fat tissue; this phenomenon would result in a clear survival advantage during periods of food scarcity, especially in our ancestral environment.[38-41] However, although modern individuals are equipped with genetic programming that is suitable for survival under any type of calorie restriction, some individuals have a greater number of these energy-saving economical genes in comparison to others, and therefore, they are more likely to develop obesity in response to a high calorie diet. Overfeeding combined with the sedentary lifestyle imposed by modern life causes the genetically predisposed to accumulate fat in central deposits in the body. This fat, which is known as visceral fat, is directly related to the development of insulin resistance and potentially to diabetes mellitus.[42]

The quality of the diet also has an important influence on the development of insulin resistance.[43] These factors modulate the insulin receptor or its signaling pathways, increase cytokine production by adipose tissue and alter the gastrointestinal production of enterohormones that are related to the hunger/satiety dichotomy and to carbohydrate metabolism.[44-46] Mitochondrial dysfunction has also been proposed to be a primary cause of insulin resistance[47,48] and the intracellular accumulation of fat.[49]

However, in addition to functioning solely as a storage compartment, adipose tissue is also currently considered an endocrine organ that secretes molecules that are protective against the development of type 2 diabetes mellitus, such as adiponectine.[50] Adipocytes also produce factors that trigger inflammatory phenomena responsible for a large number of diseases that are associated with obesity and insulin

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resistance.[51-54]

Within this multi-faceted and intensely imbricated range of factors that are involved in the genesis of obesity and its associated diseases (especially in the most important of them with respect to glucose metabolism disorders), the most prominent hormones, i.e., glucagon-like peptide-1 (GLP-1), are those produced by the digestive tract and, more specifically, those produced by L cells located in the small and large intestines and concentrated more densely in the terminal ileum.[55] GLP-1 is secreted by neuroendocrine intestinal L cells in response to the direct stimulation generated by nutrients endowed with an incretin effect.[56]

Incretins are hormones that are secreted in the gastrointestinal tract and enter the bloodstream in response to the intake of certain nutrients. This process results in an increase in insulin production and thus a rise in glucose uptake. GLP-1 has some well defined functions such as the following: stimulation of glucose-dependent insulin secretion; elevated transcription of the insulin gene, which increases the biosynthesis of this hormone; neogenesis and proliferation induction in beta cells in the islets of Langerhans; inhibition of beta cell apoptosis; increased phenotypic expression of differentiated beta cells; stimulation of somatostatin production; reduction of glucagon production.[57,58]

Diabetes mellitus type 2 (DM2) is an epidemic that affects over 150 million people worldwide. This number is expected to double in the first decades of the third millenium.[59] The therapeutic approach to DM2 and insulin resistance includes a wide variety of options, such as a multidisciplinary medical treatment aimed at weight loss, pharmacological options and bariatric and metabolic surgical techniques. The effectiveness of these techniques in controlling DM2 is well known, even over long-term durations.[60] Interestingly, bariatric interventions have also been shown to be effective for the treatment of type 1 diabetes by improving the therapeutic control of blood glucose.[61]

In contrast, the maintenance of glycemic control in obese diabetics by conservative measures remains a challenge. Diets that require a high caloric restriction and multidisciplinary clinical programs for weight loss have rarely demonstrated benefits in medium- and long-term studies. In addition, antidiabetic drugs and the long-term efficacy of insulin therapy are associated with limitations and side effects in DM2.[62,63] In this context, bariatric and metabolic surgery is presented as a valid alternative for the treatment of obesity and its comorbidities, including DM2.[64,65]

The evolution of motility studies of the digestive tract and the characterization of neurohormones (neurogastroenterology) have resulted in a better

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understanding of the features of gastrointestinal physiology. Thus, bariatric surgical methods have evolved to the current concept of mixed procedures, which also address, in addition to the features of restriction and malabsorption, the neurohormonal and metabolic factors associated with such procedures. Baroendocrine surgery and metabolic surgery are used increasingly, especially for the treatment of diabetes mellitus type 2.[66,67]

In fact, the "mixed" bariatric surgical methods comprise a more effective treatment for patients with morbid obesity; they demonstrate greater safety, sustained long-term weight loss and significant improvements in the associated comorbidities.[68] A number of studies have shown that there is na increased release of GLP-1 and better glycemic control following a Roux-en-Y gastric bypass, even before the advent of significant weight loss. These findings demonstrate that the control of diabetes mellitus may be related to hormonal effects that are secondary to the surgical technique performed.[69,70]

Although they provide greater weight loss that is sustained for longer periods, mixed techniques such as the Fobi-Capella procedure have disadvantages, especially over long durations, including dumping syndrome,[71] internal hernias,[72] anastomosis stenosis,[73] gastrogastric and enteroenteric fistulas,[74,75] peptic ulcers,[76] diarrhea, hypoproteinemia, hypocalcemia and difficulty associated with endoscopic access to areas outside the digestive tube.[77-79]

With the goal of avoiding the complications associated with the Fobi-Capella procedure, vertical gastrectomy emerged. This procedure was previously the first part of a more complex mixed operation called the duodenal switch,[80] and it involves the removal of the greater curvature and gastric fundus and a consequent reduction of ghrelin production.[81-82] Vertical gastrectomy is an emergent restrictive bariatric surgery;[83,84] it causes satiety and can be used as an initial stage in a two-stage operation for high-risk surgical patients,[85-87] or as an isolated and final surgery.[88]

Vertical gastrectomy alone has demonstrated satisfactory results in weight loss,[89] glycemic control[90] and early satiety, even in long-term assessments.[91] The achieved postoperative glycemic control may be due to the arrival at the distal ileum of food that is not fully digested. This phenomenon depends on the increased speed of gastric emptying after surgery and results in an increase in GLP1 secretion by L cells.[92,93]

Recent studies have shown that vertical gastrectomy as an isolated and definite bariatric surgery is as effective as the Fobi-Capella procedure for the control of comorbidities; in addition, it provides the potential to avoid some of the associated complications.[94-97] Although the surgical procedure is simpler, research investigating humans who were undergoing vertical gastrectomy alone demonstrated the presence of limitations[98] and complications.[99,100]

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Studies continue to be developed with the objective of developing simpler and effective surgical therapies for metabolic diseases that are associated with overweight and obese individuals, especially DM2. In this scenario, it has been postulated that anti-incretin factors may be secreted into the proximal small intestine and that these factors would no longer be produced in the absence of that intestinal segment. The absence of the alleged "anti-incretin" in circulation would enhance the action of incretins and thus improve DM2.[101-105]

Several authors claim to have demonstrated that derivation of the digestive transit to exclude a short segment of the proximal small intestine alone provides an improvement in diabetes mellitus type 2, irrespective of the effects of restricted food intake, loss of body weight, possible malabsorption of foods or the arrival of a smaller amount of digested food in the distal small intestine. Based on their results, these authors advocate surgery with duodenal exclusion as a valid alternative for the treatment of diabetes mellitus type 2. Moreover, they argue that other unknown factors or substances secreted by the proximal intestine may contribute to the etiopathogenesis of diabetes mellitus type 2.[101-105]

More recently, promising results have been reported with respect to techniques that combine vertical gastrectomy and interposition of the distal ileum in the proximal jejunum in humans. This type of surgical procedure may induce early satiety together with improved glucose metabolism and result in weight loss over short and medium durations. In non-obese diabetic patients, this surgical technique has been shown to be effective in controlling diabetes mellitus type 2.[106-111]

Interestingly, in the antidiabetic surgical interventions proposed by that team,[106-111] vertical gastrectomy was one of the prominent surgical techniques employed. Note that vertical gastrectomy is a restrictive (based on the marked reduction of the gastric reservoir capacity) metabolic procedure (based on the reduction of circulating levels of the orexigenic hormone ghrelin, which is produced by the fundus and greater gastric curvature)[82,91] that primarily aims to achieve weight loss. In this scenario, the following question arises: what is the purpose of conducting this operative step (vertical gastrectomy) in patients who do not require weight loss? One can infer that the metabolic benefits obtained by such procedures are almost exclusively due to ileal interposition. Note that the increased speed of intestinal transit caused by vertical gastrectomy[92,93] may contribute to the more rapid arrival of undigested food in the region of ileal segment interposition and thus contribute to the production of GLP-1 by L cells in the ileum. Nevertheless, the isolated ileal interposition has proven to be

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effective in correcting dysmetabolism in several animal studies.[112, 113]

It should be noted that an evaluation of surgical modalities in the context of metabolic and bariatric surgeries that involve ileal transposition alone has not yet been performed in humans. However, animal studies have demonstrated that the interposition of a segment of the distal ileum in the proximal jejunum involves an increase in the synthesis and release of GLP-1.[114] This phenomenon can be attributed to a greater stimulation of the L cells located in the interposed segment of the ileum due to a greater amount of partially digested nutrients. The facilitated arrival of nutrients in this portion of the distal small intestine causes the release of GLP-1. GLP-1 in turn stimulates an increase in the production of insulin, which directly affects glucose metabolism.[115] GLP-1 levels increase in the plasma in response to food stimuli and have a satiating effect on the central nervous system,[116] reduce the absorption of fat via the gastrointestinal tract[117] and decrease gastric[118] and intestinal[119] motility. Finally, the most notable effect of GLP-1 is the promotion of a decrease in peripheral insulin resistance, a reduction of apoptosis in pancreatic β cells and an increase in the differentiation of primitive cells in pancreatic canaliculi into adult beta cells and an increase in their proliferation,[120,121] which is known as "empty ileum syndrome".[122-124]

GLP-1 is an incretin hormone that demonstrates a strong association with the antidiabetic effects of bariatric surgery. The stimulation of ileal L cells to cleave proglucagon and release GLP-1 appears to be the most effective means of obtaining the effect of incretin in diabetic patients undergoing bariatric surgery. Several techniques can achieve this effect, all of which are based on the hindgut theory. This theory is based on the knowledge that when the ileum is contacted by food that is incompletely digested, it corrects the deleterious effects of "empty distal bowel syndrome" that are caused by a lack of L cell stimulation to produce GLP-1.

Stimulation of the ileum by undigested foods causes the release of intestinal hormones with actions that interfere positively in the control of obesity and its associated metabolic disturbances, especially in peripheral insulin resistance. The mere interposition of an ileal segment in more proximal segments of the small intestine provides results that better emphasize this hypothesis. A study of the interposed segment of the terminal ileum in the proximal jejunum in experimental animals that were subjected to a model of diet-induced obesity demonstrated significantly increased levels of GLP-1.[125] Similarly, a clinical study of a surgical technique that places an ileal segment 50 cm distal to the Treitz angle showed the same endocrine stimulation and an improvement of diabetes.[111,126]

Research demonstrating the ability of ileal food stimulation to release GLP-

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1 and cause an incretin effect is abundant in the literature. However, the histological studies investigating L cells were performed prior to the achievement of current scientific knowledge. These studies merely identify the location of these cells and indicate that they are capable of proglucagon cleavage and the release of products (GLP-1 and GLP-2).[127-129] At present, these cells are considered to have a greater role in the surgical treatment of DM2, which seeks to elucidate therapeutic measures for DM2 based on the effect of incretin. Therefore, a knowledge of the response of L cells to this effect seems to be an important step in that knowledge.[130]

A large amount of research and information in humans are available in the biomedical literature. However, in comparison with clinical studies, studies involving experimental animals in metabolic and bariatric surgery are scarce. The use of experimental animals, especially rats, in models of obesity and metabolic syndrome appear to provide greater credibility to the conclusions obtained in comparison to healthy animals. Consequently, several experimental models have been utilized.[102,131-134]

The diet-induced obesity animal model - in which obesity is achieved via modulation of the diet - appears to provide the best mimic of the human obese condition. Although no clear pattern is evident, in most cases, morbid obesity is caused by an increase of the relative amount of fat in the diet.[138-135] Although the conclusions cannot be directly extrapolated to humans, experimental research in animals is relevant because euthanasia and the *post-mortem* examination of animals can be performed; of particular importance is the histopathology of the digestive tract. Although the profile for enterohormones after bariatric surgery together with the effects of incretin in humans is known, a direct correlation with the responses of intestinal L cells has not been described in this context.

In addition to the "ileal brake" - a term used to characterize the reduced motility of the proximal gastrointestinal transit, which is associated with the enterohormone production[139-142] secondary to ileal interposition surgery - studies have demonstrated the beneficial effects of this surgical technique in the structure of the pancreas, especially in diabetic and non-obese experimental animals.[113,115] It has also been noted that in euglycemic rats, the ileal interposition improves glucose tolerance; however, the pancreas of these animals was not evaluated.[143]

Diabetes mellitus type 2 is a progressive heterogeneous metabolic disease. The pathophysiological mechanisms associated with this disease involve two main effects: peripheral insulin resistance and a progressive dysfunction of pancreatic beta cells.[144,145] These effects invariably arise when the islets are no longer capable of maintaining a hyperinsulinemia level that is sufficient to counteract the peripheral tissue resistance. Thus, the treatment is initially directed toward a reduction of weight and

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consequently insulin resistance, thus improving glucose tolerance.

Over the years, diabetes has undergone intermediate stages, where both effects can coexist, indicating association with insulin-sensitizing drugs, which are insulin secretagogue drugs. As the deficiency increases, the therapeutic combinations of oral agents may cease to be effective in the control of hyperglycemia, and therefore, insulin administration is needed to optimize the
treatment.[144,146-148]

In a given patient, it is essential to characterize his or her degree of insulin resistance according to the observed clinical condition. This strategy will determine the therapeutic orientation. None of the current therapeutics has a significant effect on the progression of this disease.[149]

In type 2 diabetic subjects, in whom obesity and insulin resistance are present in more than 80% of cases, the treatment should initially be based on a dramatic lifestyle change. It is addressed primarily to the dietary adequacy, with low-calorie diet and implementation of regular physical activity,[150] although with a tendency to crumble. However, when behavioral measures are not sufficient to achieve or maintain adequate glycemic patterns together with weight loss - as observed in most patients due mainly to a lack of compliance with these lifestyle changes and disease evolution - oral antidiabetic agents should also be utilized.[151]

At present, different classes of drugs with different mechanisms of action are available, and these drugs can be used alone or in various combination therapies.[150] Many patients do not achieve adequate glycemic control with the use of a single drug and require long-term combinations of two or more oral agents,[151-153] sometimes as a prelude to insulin therapy.

In recent years, with the goal of improving patient adherence to drug combinations, preparations have appeared on the market that contain two components in a single tablet. However, over the years, traditional treatments with oral agents are no longer effective, and consequently, glucose levels increase and metabolic control is lost. Thus, depending on the disease evolution, individuals will require the administration of insulin.[148]

The combined regimen of insulin together with oral agents may also become insufficient over time, as evidenced by an increase in blood glucose levels by self-monitoring and a progressive increase in the levels of glycated hemoglobin. Consequently, larger and more frequent daily doses of insulin are required. Finally, when it is no longer possible to use combined formulations of oral agents and insulin, full insulinization is indicated. Nevertheless, insulin is the only effective therapeutic

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alternative when conservative measures and oral antidiabetic agents are no longer sufficient for metabolic control.[144,146,154]

The intensification of insulin therapy in the treatment of type 2 diabetes is the most appropriate strategy to achieve normoglycemia and to reduce disease complications together with early, thorough, persistent and effective glycemic control.[144,155-157] Although patients undergoing intensive treatment are unable to achieve a normalization of glycated hemoglobin levels, they present a significant reduction of the risk for the onset or progression of chronic complications and late complications in diabetes (micro and macrovascular complications), which are an important cause of morbidity and mortality in the affected population.[144,155,156]

Thus, type 2 diabetes is a multifaceted condition that requires the integrated and individualized care of each patient,[150] and such care can be challenging challenge.[158] Patients often present poor blood glucose control despite the associated therapeutic measures.[149,158]

The glycemic control of an individual with type 2 diabetes is considered optimum when his or her glucose values are similar the level detected in an individual without diabetes,[159] with respect to the prevention of complications.[144] Therefore, the achievement of optimal levels of glycemic control remains a major challenge in clinical practice; only a small portion of people with diabetes attain the therapeutic goals.[157,159]

The limitations of most of the available therapeutic measures have prevented the achievement of this objective[159]. This observation may be explained by poor adherence to diet, also not so effective, and insufficient initiatives for weight loss, added to the limited efficacy of therapeutic agents associated with excessive adverse events or delay on the onset of insulin therapy and the poor acceptance of a daily regimen of multiple injections by the patient.[157,160]

It is well known that most individuals will not sustain an adequate level of glycemic control based on behavioral measures or oral monotherapy.[151] In addition, even if traditional oral therapies are usually effective in reducing hyperglycemia, they do not prevent or impede the progression of the disease, and thus, many patients require the administration of insulin.[148] Other individuals may also have allergies or contraindications to the medications, and they may not adhere or be resistant to continuity of the treatment in the presence of undesirable side effects such as weight gain and hypoglycemia, which are associated with many conventional therapies. In addition, individuals may present skin reactions, gastrointestinal disorders, hematologic reactions, acute coronary events, visual changes and metabolic diseases, which limits the use of these therapies.[147-149,152,158,161]

Most antidiabetic medications, including sulfonylurea, thiazolidinediones and insulin, improve glycemic control but do not always correct the associated

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metabolic disorders and are associated with an increase in body weight (as occurs with insulin treatment) by stimulating the appetite; this phenomenon generates concern.[147,161,162] Sulfonylurea has been shown to accelerate apoptosis in beta cells, which results in a decline of linear functions and a decrease in the mass of the human pancreas.[145] Concerning thiazolidinediones, the associated weight gain is promoted by volume expansion and edema, which can lead to heart failure in susceptible individuals or aggravate a pre-existing condition. Thus, thiazolidinediones may cause or exacerbate congestive heart failure and even ischemic cardiac events, mitigating the risks associated with the increase in body fat.[151,152,161] Recent data also suggest that thiazolidinediones can reduce mineral bone density.[151] Thus, the therapeutic strategy must consider the effects on the associated comorbidities and associated costs, in addition to the decrease in glycated hemoglobin, the tolerability and the non-glycemic effects of antidiabetic agents.[151,154]

In addition to the progressive nature of the disease itself, inadequate glycemic control is also caused by delays in the initiation of treatment with oral antidiabetics and insulin and a failure to implement the early intensification of insulin therapy in patients who are not achieving their glycemic goals.[148,155]

There are barriers for both patients and doctors who undertake the initiation and intensification of insulin therapy; these barriers correlate with poor individual motivation, a lack of familiarity with application, the need for frequent insulin injections, fear that the injection will be painful and difficult to administer and concerns regarding hypoglycemia and weight gain.[155,160,163] Consequently, an inappropriate delay in the initiation of early insulin therapy prevents the achievement of the recommended glucose levels and the maintenance of adequate glycemic control.[146,154,160]

To achieve a successful insulin therapy, the patient must be adequately informed and motivated by the participation and support of a multidisciplinary team. At present, several insulin preparations are available for the treatment of diabetes, including different pre-mixes and cartridge preparations that are applied via pen injectors. These techniques can increase the complexity of the treatment of diabetes. The patient should be instructed with respect to the various presentations, forms, routes and sites of drug application. The storage and care of insulin and the importance of monitoring blood glucose should also be explained. Furthermore, the need for other lifestyle changes, such as a proper diet and physical activity level, must be emphasized. The risk for the development of hypoglycemia should also be explained to the patients and their families, together with the measures necessary to prevent and reverse this condition.

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Thus, the intensive insulin treatment scheme requires a higher level of socio-economic and cultural development in the patient, a high level of compliance and the ability to learn; the patient will be monitoring the treatment and determining the insulin rates, the presence of hypoglycemia and the prevention of acute complications. The required amount of knowledge is rather large, and the patients must be aware of the availability of resources to implement the treatment protocol. In addition, both the patient and the entire multi-professional medical team should be involved in the treatment.

Thus, there is a clear need for new, more aggressive antidiabetic therapeutic options, which could be combined with the existing pharmacological agents to preserve the functions of beta cells and halt the progression of diabetes mellitus type 2.[147] None of the mentioned available therapies for diabetes mellitus have been shown to preserve the functions of pancreatic beta cells over time. The limitations of this treatment demonstrate the importance of discovering new resources that offer greater efficiency, durability, convenience, safety and tolerability to achieve the goals of early adequate glycemic control and the prevention or delay of the need for additional measures.[145] More aggressive options are therefore required based on the pathogenesis of this disease, and such options should include a greater control of both fasting and postprandial glucose.[154]

Incretin-based therapies such as GLP-1 receptor agonists administrated subcutaneously and DPP-4 inhibitors (dipeptidyl peptidase-4) administered orally offer a new, more current and interesting approach in the management of type 2 diabetes, and the development of new agents is currently underway.[145,149] The two groups that are presently available have been demonstrated to be safe and effective in reducing blood glucose levels while also promoting a favorable effect on weight reduction and maintenance.[148,151,161,162] Some studies have demonstrated the beneficial effects of these promising drugs on the lipid profile and blood pressure in patients.[145]

Agonists of the GLP-1 receptor and inhibitors of DPP-4 both lead to the secretion of insulin and the suppression of glucose-dependent glucagon, with a consequent low risk for hypoglycemia when used alone or in combination therapies, excluding the concomitant use of sulfonylurea. Moreover, experimental studies have demonstrated a prolonged survival of pancreatic beta cells, with a delay in their dysfunction and a promotion of their regeneration. Theoretically, these phenomena permit a possible decrease in the progression of type 2 diabetes mellitus. These results

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have not yet been verified in clinical studies.[145,148,149]

However, despite the attractive features of these new agents, their high cost in comparison to first-line oral drugs and the lack of long-term data concerning their safety and clinical outcomes should be considered. There have been reports of nausea, headache and cases of acute pancreatitis, upper respiratory infection, depression, hypoglycemia and severe allergic skin reactions associated with the use of these drugs; some individuals also exhibit a modest reduction (approximately 1.0%) of glycated hemoglobin (HbA1C) levels in comparison to the levels detected in patients treated with insulin and older agents. The development of carcinomas in guinea pigs in response to new agents has not yet been confirmed in humans.[145,152,153]

In addition to the new classes of antidiabetic medications, the development of insulin analogues and other non-invasive alternatives to the use of insulin are promising options for the management of diabetes mellitus type 2. [146,163] Such agents may assist in overcoming some of the challenges associated with this disease to achieve enhanced glycemic control, despite the difficulties associated with the natural disease progression and the loss of efficacy related to the use of long-term oral agents.[159]

Novel basal insulin analogues, including pre-mixes such as lispro, aspart and glargine, represent a more physiological replacement to previous therapies because they provide greater freedom, flexibility and convenience with respect to the drug administration. Concerning the content of the diet, they provide a better quality of life compared to regular insulin formulations.[144,146,163] Innovative formulations without the need for subcutaneous injections can overcome some of the shortcomings associated with typical insulin therapy and thereby facilitate the early use of insulin. These effects allow the achievement and continuance of long-term glycemic control, which enhances quality of life.[157] Moreover, there is a clear need to include appropriate types of counseling with individual education in the modern therapies available for the clinical treatment of type 2 diabetes to overcome the known barriers related to insulin therapy.[154]

The objectives of the treatment of diabetes mellitus type 2 are to improve the quality of life and prevent early mortality in patients through glycemic control, which minimizes the risk for microvascular effects; however, substantial benefits regarding macrovascular implications have not been demonstrated.[62,164-167]

Meanwhile, the maintenance of adequate glycemic control in obese patients with type 2 diabetes using conservative techniques remains a real challenge. Diets that require a large calorie restriction and multidisciplinary clinical weight loss programs

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have rarely demonstrated significant benefits over medium and long-term periods that extend beyond their own limitations and the side effects associated with antidiabetic agents and insulin therapy. To date, even intensive schemes for glycemic control that combine and optimize the institution of traditional agents have failed to achieve these therapeutic goals. In addition, the new oral agents and insulin analogues, which require further studies for validation, consider the individual needs and lifestyle of each patient. Thus, in addition to significant glycemic control, measures concerning the lifestyles of diabetic patients with respect to the reduction of obesity, hypertension, dyslipidemia and smoking must be considered to achieve the desired goals in the treatment of type 2 diabetes.[62,145,154,168]

In the therapeutic management of DM2 and insulin resistance, in addition to a wide variety of multidisciplinary clinical treatment options, which aim to achieve weight loss and glycemic control using pharmacological agents, bariatric and metabolic surgical techniques may be important in this treatment regime. It is well known that these surgical techniques can control type 2 diabetes, even over long-term periods.[169] The surgical treatment of patients with diabetes mellitus type 2, irrespective of obesity, must currently be considered as a valid alternative approach to the treatment of this disease.

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2. OBJECTIVES

2.1 General

To evaluate the physiological effects of ileal transposition in metabolic syndrome.

2.2 Specific

2.2.1. To investigate the morphological and functional changes that occur in intestinal L cells in a segment of the distal ileum interposed to the proximal jejunum in rats with diet-induced obesity.

2.2.2. To study the structural changes in the small intestine of animals subjected to the transposition of a segment of the distal ileum to a proximal location in the digestive tract in rats with diet-induced obesity.

2.2.3. To quantify and evaluate the morphology of endocrine cells in the pancreas after ileal transposition in rats with diet-induced obesity.

2.2.4. To verify the changes in the metabolism of glucose and lipids in rats with diet-induced obesity that are submitted to ileal transposition.

3. BACKGROUND AND EXPECTED OUTCOMES

The surgical intervention studied herein – the interposition of the ileal segment in the proximal jejunum - could become a valuable therapeutic approach for the control of diabetes mellitus type 2, even in non-obese individuals, because it does not affect the absorption of nutrients. The evaluated surgery is simple to execute, inexpensive, can be easily reversed and does not require removal of the digestive tube segment or result in food transit deviations. Thus, this technique avoids the incidence of malnutrition and other complications.

However, there is the need to investigate the possibility that with a long-term, constant exposure to high levels of digestive juices from the stomach, pancreas and liver and the constant contact between the transposed ileal mucosa and the incompletely digested food, the mucosa of the ileum will become "jejunized", i.e., it will present histological cell features, membrane structures of absorption and exocrine and endocrine secretion patterns similar to those associated with the jejunum. This potential "jejunization" of the ileum is of interest only with respect to exocrine and absorptive components (which cause no problems) or changes in the ileal endocrine pattern (a high density of GLP-1-producing L cells) toward that of the jejunum (a low density of these cells). This last circumstance will invalidate the efficiency of the proposed technique in the long term. It is noteworthy that if the present research is rejected, the theoretical possibility that the epithelium of the mucous membrane of the interposed ileal segment in the proximal jejunum acquires a jejunal phenotype - including in particular the low density of L cells typical of the proximal jejunum - would be the only theoretical obstacle to the achievement of ileal interposition in diabetic individuals. Thus, if the interposed ileum maintains a high density of L cells, the long-term action (mainly toward the endocrine pancreas) of the well studied beneficial trophic effects of GLP-1 will be guaranteed.

Once such intervention has been studied in the context of translational medicine in animals, which demonstrate physiological characteristics that are very similar to those of humans, with respect to glucose metabolism and nutrition in general. The results of this study could be extrapolated to clinical scenarios. The relevance of this research lies in the benefits that the studied surgical intervention could provide to a

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large population of diabetic patients, even non-obese patients that present substantial control and even remission of dysglycemia.

The use of immunohistochemistry of the intestinal tissue is expected to demonstrate the preservation of the high density of L cells in the interposed ileum, despite the anticipated change in the mucosal epithelium with respect to exocrine secretion and absorption from the ileal to the jejunal pattern. This hypothesis is based on the knowledge that the differentiation of stem cells in the crypts toward L cells is not affected by epigenetic modifications. Researchers hope that the stem cells in the crypts of the fundus will continue to differentiate normally into L cells. These stimuli are known to induce the transformation of the ileal secretory exocrine and absorptive epithelial patterns in the mucosa of the jejunum; however, it remains unknown whether this "jejunization" also involves the transformation of the high density of L cells in the normal ileum to a low density of these cells in the transposed ileum. Immunohistochemistry of pancreatic tissue should demonstrate the preservation of the islets of Langerhans, insulin-producing cells and proliferating cells. Glucose intolerance and insulin resistance should diminish or disappear in the group undergoing ileal transposition. Some weight reduction in the absence of malnutrition should be observed in the animals undergoing ileal transposition, and no associated adverse effects are expected.

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5. METHODS

Experimental Model

This research project was approved by Experimental Research Ethics Committee of the Federal University of São Paulo - Escola Paulista de Medicina (Universidade Federal de São Paulo - UNIFESP/EPM).

Sample

The sample includes 40 male rats (Rattus norvegicus albinus, Wistar 2BAW, heterogeneous) aged 12 weeks with a body weight ranging from 250 to 280 g. The animals will be supplied by the Laboratory of Animal Experiments at the Pharmacology and Molecular Biology Institute (Laboratório de Experimentação Animal do Instituto de Farmacologia e Biologia Molecular, Universidade Federal de São Paulo, Escola Paulista de Medicina (UNIFESP/EPM)), where they will be maintained throughout the experimental procedure.

The animals will be housed in individual cages for 16 weeks with a controlled room temperature of 23 ± 2°C, a relative humidity of 55 ± 15% and an automatic timer (Kienzle) that provides 12 hours of alternating light and dark cycles (06:00/18:00).

The animals will be randomly divided into four groups according to the chart below:

- transposition group (TG) - animals submitted to ileal transposition, 10 animals that receive a hypercaloric-hyperlipidic diet;

- sham group (SG) - animals submitted to the sham surgery, 10 animals that receive the same diet;

- control group 1 (CG1) - animals that are not submitted to any surgical intervention but receive a hypercaloric-hyperlipidic diet;

- control group 2 (CG2) - animals that are not submitted to any surgical intervention or a hypercaloric-hyperlipidic diet but receive only standard feed, 10 animals.

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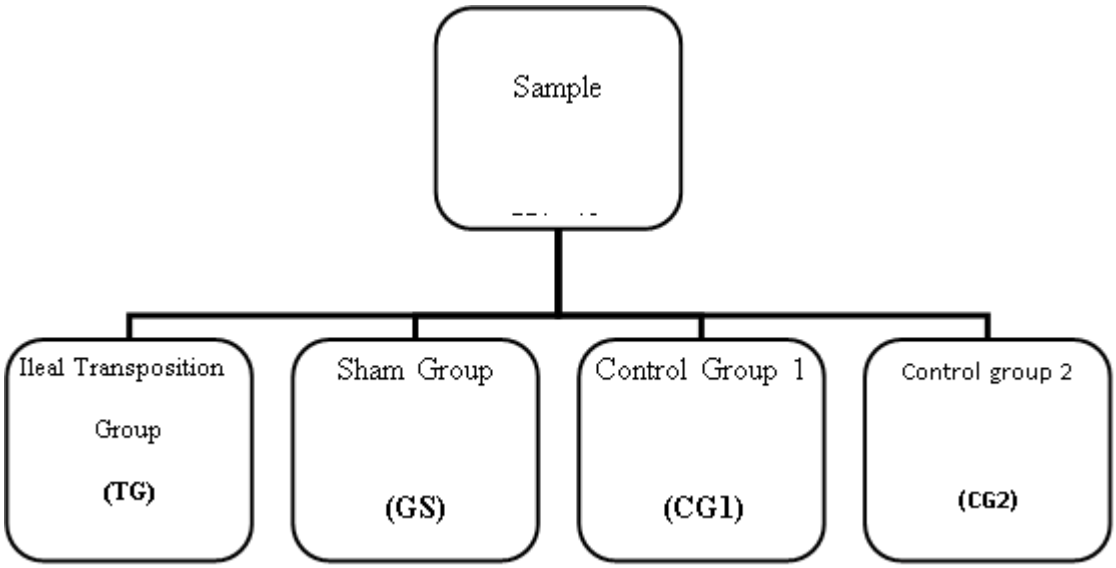

The surgeries will be performed in 20-week-old animals. All of the animals will be monitored until the 8th postoperative (PO) week, i.e., the 28th week of life, at which time they will be sacrificed.

Experimental Procedures

The experimental procedures will be performed in accordance with the guidelines of "Cuidados e Manejos de Animais de Laboratório," Ed: Lapchick, Mattaraia, Ko; Atheneu, 2009, CDD 636.0885.

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Diet

The rats will be randomly allocated to the TG, SG and CG1 groups and maintained on a high calorie diet (DH) that is rich in lipids, pelletized and provided *ad libitum* with alternate cycles of four different types of food (1, 2, 3 and 4) for a period of 16 weeks to stimulate food intake. The experimental diets will alternate every 24 hours, and the amount of food that has not been consumed will be measured. The consumption of these diets will promote obesity in animals, which will result in features that are commonly associated with human obesity, such as insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia and hepatic steatosis. The animals in group CG2 will receive a standard diet. All of the groups will have unlimited access to water.

The experimental diets 1, 2, 3 and 4, according to the specifications established by the *Nutrient Requirements of the Laboratory Rat* recommended by the *National Academy of Sciences*, will be industrially produced by Nuvital®, São Paulo - SP. These diets will consist of standard Nuvital® feed for rats, which contains protein and vitamin and mineral supplementation. The additional hyperenergetic ingredients included in the preparation of the experimental hypercaloric diets are (in grams per kilogram):

- DH1 - Nuvital® standard feed, 355; roasted peanuts, 176; casein, 123; corn oil, 82; chocolate, 88; corn biscuit, 176; vitamins and minerals.

- DH2 - Nuvital® standard feed, 439; roasted peanuts, 218; casein, 129; corn oil, 61; potato chips, 153; vitamins and minerals.

- DH3 - Nuvital® standard feed, 371; roasted peanuts, 185; casein, 99; corn oil, 68; noodles, 185; grated cheese, 92; vitamins and minerals.

- DH4 - Nuvital® standard feed, 359; roasted peanuts, 179; casein, 105; corn oil, 80; condensed milk, 161; wafer biscuit, 116; vitamins and minerals.

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The macronutrient composition of the standard and experimental hypercaloric-hyperlipidemic feeds, which were produced and analyzed by Nuvital®, São Paulo - SP, is shown in Table 1.

Table 1 - Composition of standard and experimental diets.

	Feeds				
Components	Pattern	DH1	DH2	DH3	DH4
Protein (%)	26	27	28	28	26
Carbohydrates (%)	54	43	36	33	43
Fat (%)	3	20	23	24	20
Others (%) †	17	10	13	15	11
Calories (kcal/g)	3.5	4.6	4.6	4.6	4.6

† - vitamins, minerals, moisture, ash.

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Weight and Feed Intake Curves

The animals will be weighed twice a week on pre-established days. Weighing will be performed at the start of the light period using an appropriate precision scale (Filizola BP-6), and the weight of each animal will be recorded in grams.

Feed intake will be measured three times a week on pre-determined days by calculating the difference between the weights of the feed provided and that consumed in each cage. These results will be calculated in grams.

Weight and feed intake curves will be generated for all of the groups throughout the experiment.

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Fasting

Animals in all of the groups will be offered only water during the six hours prior to the collection of blood for laboratory tests and surgical procedures.

Blood Collection and Laboratory Tests

Blood samples will be collected for laboratory testing from 12-week-old animals (W12), on the day of surgery (S20) and eight weeks after the surgery or at 28 weeks of age (S28). For this procedure, the animals will be weighed and anesthetized.

The blood collection on day zero (D0) will be performed to obtain the baseline parameters of each animal, whereas that performed on the day of surgery will determine changes in glucose metabolism that are secondary to the obesity induced by the hypercaloric-hyperlipidemic diets. The blood collection at eight weeks after surgery will be performed to evaluate changes in the same metabolic changes following the surgeries.

One milliliter of blood will be collected into heparinized tubes by puncturing the tail vein of each animal, followed by centrifugation and subsequent storage at -20°C for the determination of blood parameters (LABTEST®), including glucose (GLU), triglycerides (TG), total cholesterol (COL) and the fractions of low-density lipoprotein (LDL) and high-density lipoprotein (HDL); the levels of insulin (INS), glycated hemoglobin (HBG), C-peptide (CPT) and glucagon-like peptide-1 (GLP1) will be evaluated by ELISA (Linco®). After 10 minutes of blood collection, the serum glucose level will be determined with reagent strips using a glucometer (Accu-Chek Advantage Kit®) followed by the insulin tolerance test (ITT), for which a solution of regular insulin at a concentration of 1 U/kg body weight will be injected through the penile vein. Blood samples will then be collected to determine the levels of glucose. A lancet will be used to obtain a drop of blood from the tail of each animal at 4, 8, 12 and 16 minutes after the injection of insulin. The results will be recorded for later analyses.

Anesthesia, Weight Assessment and Antibiotic Prophylaxis

Prior to anesthesia, all of the animals will be weighed using a precision scale (Filizola BP-6), and their weights will be recorded in grams. In

both groups TG and SG, general anesthesia will be induced with halothane to collect blood from the tail. Subsequently, a dissociative general anesthetic will be used to perform the insulin tolerance test and surgery: Zoletil 50® (tiletamine + zolazepam) at a dose of 20 mg/kg and fentanyl® at a dose of 0.025 mg/kg, collected in the same syringe and administered simultaneously via an intramuscular injection.[170]

Each animal will be maintained under spontaneous ventilation during the procedures, and anesthesia will be monitored through regular evaluations (every 45 minutes) of the auricular and interdigital reflexes, which should be abolished in anesthetized animals. If such reflexes are observed, complementary doses that comprise one third of the initial dose will be administered. After completion of the surgery, the total amount of anesthetic used will be recorded.

The antibiotic prophylaxis will comprise an intramuscular injection of 50 mg/kg cefoxitin immediately after the surgical anesthesia.

Operative Procedures

On predetermined days, two 20-week-old (S20) rats will be distributed randomly into TG and SG. The aseptic and antiseptic techniques associated with Halsted's principle will be performed using sterile micro-surgical instruments.

The hairs on the abdominal region of anesthetized animals will be cut close to the skin with scissors while the animals are in a supine position on the operating table with their paws and tail properly grounded and secured with adhesive tape. Antisepsis of the abdominal region will be performed using chlorhexidine suspended in an aqueous vehicle.

A fenestrated sterile surgical field will be placed in the abdominal region of the animal, and a median incision of approximately 5 cm in length will be generated in the abdominal wall using a disposable scalpel (blade number fifteen).

Initially, the cecum will be identified and exposed together with the terminal ileum. Subsequently, in both groups, the small intestine will be sectioned transversely in regions that are 3 and 5 cm distant from the ileocecal transition. In addition, a 2-cm ileal segment will be resected for the histological study.

In the TG animals, the ileum will be resectioned transversely at a distance of 15 cm from the ileocecal transition to separate an ileal segment of 10 cm, which will be wrapped in gauze that has been previously moistened with heated 0.9% sodium chloride

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solution (Figure 1A). This procedure will be followed by anastomosis of the remaining ileal segments to restore the continuity of the digestive tract. Subsequently, the jejunum will be sectioned in two places (a distance of 5 and 6 cm from the duodenum-jejunum transition), and a 1-cm segment of the jejunum will be resected to perform anatomical and pathological studies (Figure 1A). The segment of distal ileum will be interposed to the previously separated segments of the sectioned jejunum in a isoperistaltic position via entero-enteroanastomosis (Figure 1B).

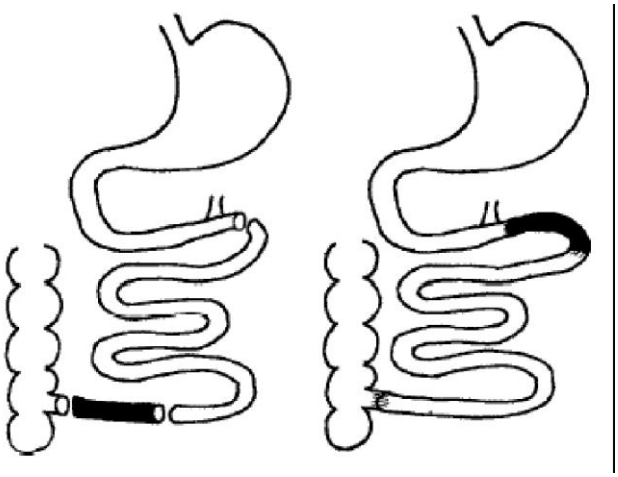

Figure 1A Figure 1B

Figure 1A - Schematic of the regions of the intestinal section and the isolated ileal segment (filled in black) implemented in the procedure.

Figure 1b - Diagram showing the previously anastomosed distal ileum and transposed anastomosed ileal segment (filled in black) in the initial segments of the jejunum.

In SG animals, the intestine will be sectioned transversely in regions located at a distance of 15 cm from the ileocecal transition and 5 and 6 cm from the duodenal-jejunal transition, following the making of primary intestinal anastomosis, without transposition, after the same jejunal and ileal resections for study similar to the TG.

All of the intestinal anastomoses will be termino-terminal and performed at six separate points using a 7-0 polypropylene thread that is pre-assembled in a cylindrical needle.

After the final revision and completion of the surgical procedure, the animals will be hydrated via an intraperitoneal injection of 1.0 mL of crystalloid solution (0.9% saline solution) for every 300 g of body weight at 36ºC.

Synthesis of the abdominal wall will be performed by continuous suturing in

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a monobloc of the parietal peritoneum, the muscular layer and the aponeurosis using a 4-0 polyglactin thread that is pre-mounted in a cylindrical needle. A continuous suture with inverted points will be generated in the skin using a 4-0 polyglactin thread that is pre-mounted in a cylindrical needle.

Postoperative treatment

The animals will be kept warm and observed until complete recovery from the anesthesia. Subsequently, they will be placed in individual cages and transported to the mouse facility in the same laboratory under the same pre-operative environmental conditions.

Prior to the recovery from anesthesia, analgesia will be applied by gavage with 0.5 mL of a solution containing 3 g of dipyrone dissolved in 1 mL of water. Water will be reintroduced *ad libitum* water immediately after recovery from the anesthesia. During the first 72 hours after surgery, the rats will be maintained with 1 g dipyrone diluted in 100 mL of water. They will be permitted access to the diet for 12 hours after completion of the surgery.

The animals will be evaluated and monitored until the 8^{th} week after surgery, i.e., the 28^{th} week of life (S28), which is equivalent to a five-year postoperative period in humans.[171]

Euthanasia

In 28-week-old animals, the same fasting, anesthetic, weighing and blood sampling procedures for laboratory tests will be performed followed by decapitation, according to guidelines of the Sociedade Brasileira de Ciência de Animais de Laboratório/Colégio Brasileiro de Experimentação Animal – SBCAL-COBEA.

Body Composition

Following euthanasia, the fat deposits in the periepididymal and retroperitoneal regions will be collected and weighed using a precision scale. This procedure will be conducted for all of the animals in each group to quantify the amount of adiposity.

Collection of Pancreatic Tissue

Following euthanasia, the pancreas will be collected from animals in each groups for the pathological and immunohistochemical studies. The organ will be resected in a monobloc near the large gastric curvature and the splenic region.

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Collection of Intestinal Tissue

Upon completion of the surgery in SG and TG, tissue will be collected for the initial histological evaluation from the following regions:

- distal ileal segment - I 1 (ileum, at time 1);

- proximal jejunal segment - J 1 (jejunum, at time 1).

Following euthanasia, the following intestinal tissues will be collected in all of the groups for a second analysis of the structural morphology of the intestine and the intestinal cells that produce incretin hormones (L cells) as well as their possible long-term modifications:

- ileal segment, distal to the ileoileal anastomosis - I 2 (ileum, at time 2).

- jejunal segment, distal to the ileojejunal in TG and jejunojejunal in SG and about 7 cm from the duodenaljejunal transition in GC1 and GC2 - J 2 (jejunum, at time 2);

- median ileal segment transposed in the TG and its corresponding segment in the SG, 8 cm from the ileocecal transition - T 2 (transposed segment, at time 2).

Collection of Liver Tissue

Following euthanasia, the liver from each animal will be resected, weighed on a precision scale and subjected to a histopathological analysis.

Histology - Optical Microscopy and Immunohistochemistry

Pancreatic tissue:

The pancreas of each animal will be dried in a monobloc for an evaluation using optical microscopy. The tissue will be properly positioned in a cassette, fixed in 10% buffered formaldehyde for 12 hours and then processed for paraffin embedding. Histological sections will be obtained using a Minot-type microtome set to a thickness of 5 □m and placed along the long axis of the tissue on slides that have been previously treated with 5% silane.

The slides will be stained with hematoxylin-eosin for the morphometric analysis of the pancreas. Specific antibodies will be used for immunohistochemistry:

- polyclonal antibody H-86:SC-9168, which marks insulin-producing cells (Santa Cruz Biotechnology).

- monoclonal anti-rat antibody to assess the proliferation marker Ki67, clone MIB-5, code: M7248-1, DAKO.

Intestinal tissue:

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The removed intestinal segments from each animal will be opened in the contra-mesenteric region and carefully washed with saline solution for evaluation by light microscopy. Next. they will be arranged on a piece of filter paper with the maintenance of direct contact with the serosa and with the mucosal face directed upward. The segments will then be placed in a wide mouth jar in 10% buffered formaldehyde at 10 times the tissue volume for 12 hours. During this time, the mucosal face will remain in direct contact with the solution. Subsequently, processing for paraffin embedding will be performed. Histological sections will be obtained using a Minot-type microtome set to a thickness of 5 □m and placed on slides (using the inside perimeter of the region) that have been previously treated with 5% silane.

The slides will be stained with hematoxylin-eosin for morphometric analyses of the intestine in all of the resected segments. Specific antibodies will be used for the immunohistochemistry study:

- anti-GLP-1 sc7782 antibody, clone C-17, 1:500 dilution (Santa Cruz Biotechnology).
- anti-rat monoclonal antibody to assess the marker of cell proliferation Ki67, MIB-5 clone, code: M7248-1, DAKO.

Liver tissue:

After weighing the liver of each animal, it will be macroscopically sectioned along the long axis of the organ with the hilum side facing downward. Two fragments measuring 1.5x1.5x0.5 cm each will be obtained from each liver lobe and fixed in 10% formalin for 12 hours. Next, both fragments will be processed in a single paraffin block. Histological sections will be obtained using a Minot-type microtome set at a thickness of 5 □m and placed on slides that have been previously treated with 5% silane.

Standard techniques will be used for hematoxylin-eosin and Masson trichrome staining of the slides for the histopathological analysis and staging.

Immunohistochemistry protocol:

The slides containing the pancreas fragments will be double-stained using the EnVision G/2 Doublestain System (Rb/Mo (DAB+/Permanent Red), code: K5361-1, Dako). The results will be visualized using peroxidase (HRP) and alkaline phosphatase (AP) with DAB+ and Permanent Red as the chromogen, respectively.

The slides will be assessed using immunohistochemistry by double-staining with antigen using the LSAB Kit. The results will be visualized using peroxidase (HRP) with DAB+ as the chromogen.

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The following protocol will be used for the histological sections:

a) Dewaxing: the blades will be left in an incubator at 60°C for 12 hours to obtain optimal tissue adhesion and then dewaxed in three xylene baths of five minutes each at room temperature;

b) Hydration: the blades will be submerged twice in absolute ethanol for five minutes and washed with running water for two minutes for hydration;

c) Antigen Retrieval: the slides will be placed in a sodium citrate solution of 10 mM, pH 6.0 for 30 minutes in a steam pan (95ºC), allowed to cool to room temperature for 20 minutes and washed in 0.05 M PBS (phosphate-buffered saline), pH 7.4 four times for three minutes each;

d) Blocking of Endogenous Peroxidase: the slides will be incubated with 3% hydrogen peroxide four times for five minutes each and then washed with running water and PBS, pH 7.4 three times for three minutes each;

e) Blocking of Nonspecific Sites: the slides will be incubated with PBS, pH 7.4 + 1% BSA (bovine serum albumin) for 30 minutes at room temperature;

f) Primary Antibody: histological sections of each slide will be incubated with the primary antibody SC-9168 to mark the insulin-producing cells in pancreatic tissue and SC-7782 to mark L cells in intestinal tissue. The antibodies will be diluted in PBS + 1% BSA according to the titration recommended by the supplier, and the slides will be incubated in a moist chamber at 4°C for 16-18 hours and then washed with PBS, pH 7.4 3 times;

g) Secondary Antibody: the sections will be incubated with the secondary antibody conjugated to biotin provided in the DAKO EnVision Kit for pancreatic tissue and in the Dako LSAB Kit for intestinal tissue. The incubation will be performed for 30 minutes at room temperature in a moist chamber. Next, the slides will be washed three times with PBS, pH 7.4 and then incubated with the amplification solution (HRP-conjugated streptavidin) provided in the Dako kit for 30 minutes followed by three washes with PBS, pH 7.4;

h) Visualization: the slides will be incubated with 3,3' diaminobenzidine solution (liquid DAB) for 15 minutes and then washed with distilled water;

i) Blocking of Double Staining: the pancreatic and intestinal tissue sections will be incubated with the double staining blocking solution for three minutes and then washed with PBS. The sections will then be incubated with the endogenous enzyme blocking solution provided in the Dako EnVision Kit and washed with PBS;

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j) Second Primary Antibody: the histological sections will be incubated with the second primary antibody, M7248-1, to assess cell proliferation. The antibody will be diluted in PBS + 1% BSA using the titration recommended by the supplier, and the slides will be incubated in a moist chamber at 4°C for 16-18 hours and then washed three times with PBS, pH 7.4; k) Second Secondary

Antibody: sections of both pancreatic and intestinal tissues will be incubated with the second secondary antibody conjugated provided in the Dako EnVision Kit for 30 minutes at room temperature in a moist chamber. Following the incubation, the slides will be washed in PBS, pH 7.4 three times, incubated with the amplification solution (streptavidin conjugated to alkaline phosphatase) provided in the Dako kit for 30 minutes and then washed three times with PBS, pH 7.4;

l) Visualization: the sections will be incubated with the Permanent Red chromogen solution for 20 minutes;

m) Counterstaining: the sections will be washed under running water for five minutes and then counterstained with Harris hematoxylin for 20 seconds;

n) Dehydration and Mounting: the slides will be washed with water for 10 minutes and immersed in absolute ethanol four times and then in xylene three times. The slides will then be coverslipped using Etellan® mounting medium and visualized.

Histological sections of the pancreas and ileum from one animal in the control group will be used as an internal positive control in the immunohistochemical studies, as appropriate. The omission of the respective primary antibodies during the histological procedures will serve as a negative control.

For the pancreatic tissue, the appearance of a brown color will indicate positive staining using the DAB+ chromogen, and the presence of a red color will demonstrate positive staining with the Permanent Red chromogen. For the intestinal tissue, the presence of a brown color will indicate positive staining. Immunoexpression will be evaluated by image representation using a computerized system that consists of a light microscope (Carl Zeiss) adapted to a high-resolution camera (Carl Zeiss AxioCam MRc) and color video monitor (Samsung). The images will be obtained using the Carl Zeiss image analysis software AxioVision REL 4.2.

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Study Parameters

Weight: Immediately after initiation of the light cycle, the weight of each animal from each of the four groups (TG, SG, GC1 and GC2) will be determined using a precision scale and recorded in grams (P1, P2, P3, .., P32).

Feed consumption: All of the animals will receive a known quantity of feed that is sufficient for their daily needs. The remaining feed will be weighed using an appropriate scale three times a week, and the difference between the amount of feed supplied and that remaining will be used to calculate the daily feed intake.

Biochemical tests: The amounts of serum glucose (GLU), insulin (INS), triglycerides (TG), total cholesterol (COL) and its fractions (HDL and LDL), glucagon-like peptide-1 (GLP-1), peptide C (PTC) and glycated hemoglobin (HBG) will be determined in all of the animals. These levels will be assessed at the beginning of the procedure (12 weeks of age), during surgery (20 weeks of age) and at the time of euthanasia (28 weeks of age), which will be denoted as times 1, 2 and 3, respectively.

Insulin resistance: Insulin resistance (IR) will be calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) indirect test, and this value will be calculated based on the product of serum insulin (mU/mL), glucose (mg/mL) and the constant 0.05551 divided by 22.5. This calculation will be performed for times 1, 2 and 3.

Insulin tolerance test (ITT): ITT will be performed for all of the animals at the beginning of the experimental procedure and at the time of surgery and euthanasia (times 1, 2 and 3). The rate of glucose disappearance is calculated using the formula In2/t1/2, and the t1/2 of glucose is calculated based on the linear phase of decline in the plasma glucose concentration determined in the minimum regression curve using PRISMA software.

Body fat: On the day of euthanasia, periepididymal and retroperitoneal fat deposits will be collected from all of the animals in each group and weighed on a precision scale. The adiposity index will be calculated based on the proportion while considering the body weight of each animal.

Liver weight: On the day of euthanasia, the resected liver from each animal will be weighed using an appropriate scale, and the values will be recorded in grams.

Histopathological Analysis - Interpretations of the Optical Microscopy and Immunohistochemistry Results:

Pancreatic tissue:

Hematoxylin-eosin-stained slides will be used for the morphometric analysis of the pancreas; the area comprising the islets of Langerhans and the number of islet cells will be determined. Double labeling via immunohistochemistry will be used to determine the number of insulin-producing and proliferating cells.

Images of the histological sections of the pancreas will be scanned and analyzed using ImageJ software. The total pancreatic tissue will be measured in each rat; this represents all of the fields captured in an increase of 400 times the greatest cut diameter of tissue obtained. The islets will then be distinguished for counting and measurements of their area. A relationship will be established between the total area of the islets and the total area of the pancreatic tissue. The mean area of the islets and average number of islets will be determined. In each islet of Langerhans, the following cells will be counted: cells stained for insulin, unstained cells, proliferating cells and proliferating cells stained for insulin. A relative value among labeled, unlabeled, proliferating and labeled proliferating cells will be established.

Intestinal tissue:

By staining with hematoxylin-eosin, the intestinal mucosal cells will be examined by global microscopy to determine differences in the shape, structure, size and number of the cells. The number of villi and crypts will be counted, and the height of 10 villi and 10 crypts from each intestinal segment will be measured to establish a ratio. The total thickness of the wall will also be measured.

Enteroendocrine cells related to the GLP-1 hormone (L cells) will be identified by immunohistochemistry and evaluated in relation to their structure, shape, size and number. Next, we will count and evaluate the general characteristics of the L cells. Subsequently, its density will be determined based on the ratio of the number of L cells to the area of the mucosal tissue (crypts and villi). Double labeling via immunohistochemistry will also be performed to count the number of L cells undergoing proliferation.

Liver tissue:

Histological sections stained with hematoxylin-eosin and Masson's trichrome will be analyzed according to the international modified parameters of Kleiner and Brunt, which are used to stage the degree of nonalcoholic fatty liver

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disease, according to the NAS - nonalcoholic fatty liver disease (NAFLD) activity score of the Pathology Committee of the Clinical Research Network.[172,177]

Macrovesicular steatosis will be classified as follows: grade 1 - a mild attack with less than 33% of the hepatocytes affected; grade 2 - a moderate with 33% to 66% of the hepatocytes affected; grade 3 - a severe with more than 66% of the hepatocytes affected.

Microvesicular steatosis will be classified as absent or present, and the associated localization in the acini will be recorded.

Hepatocyte ballooning will be considered absent, occasional or frequent, and the location of this event will be recorded.

Lobular inflammatory activity will be classified as absent, mild (1-2 foci, 20-fold magnification), moderate (3-4 foci, 20-fold magnification) and severe (>4 foci, 20-fold magnification).

The portal inflammatory infiltrate will be classified as absent, mild, moderate and intense based on the phenotypic characterization. In the absence of lobular activity, the result will be considered only as steatosis.

The degree of fibrosis will be classified as follows: absent, grade 1 - fibrosis limited to the perivenular area; grade 2 - perivenular fibrosis with minimal septation; grade 3 - alterations of the lobular architecture with septation and incipient nodular formation; and grade 4 - cirrhosis.

A diagnosis of nonalcoholic steatohepatitis will be established according to the American Association for the Study of Liver Diseases, and it will require the presence of steatosis, a mixed infiltrate and/or hepatocellular ballooning and/or pericellular fibrosis in the center-lobular area.[178-179]

Statistical Analysis

The variables will be summarized per study group according to the relevant descriptive statistics: absolute frequency (n) and relative (%) or mean, standard deviation (SD), median and minimum and maximum values. The data will be analyzed using parametric or nonparametric tests depending on the observed distribution.

For a normal distribution of the data, analysis of variance with two fixed factors will be applied: groups (four levels: TG, SG, CG1 and CG2); evaluation (three levels: week 12 (W12), week 20 (W20) and week 28 (W28)). For variables that are measured weekly, the analysis will comprise 16 levels according to the week (week 1 (W12); week 2 (W13), week 28 (W16)). In all of the other instances, the Mann-Whitney

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test will be applied to compare techniques within each assessment, and the Friedman test will be used for related samples to compare assessments within each technique. The presence of an association between qualitative variables will be evaluated using the chi-square or Fisher's exact test. The significance level will be established as 0.05 (α=5%), and descriptive levels (p) below this value will be considered significant.

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