# Involvement of M1 and M3 receptors in isolated pancreatic islets function during weight cycling in ovariectomized rats

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| Complete List of Authors: | Pacher, Kayo; Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, FHO/UNIARARAS
Camargo, Thaís Furtado; Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, FHO/UNIARARAS
Andrade, Thiago Antônio Moretti; Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, FHO/UNIARARAS
Barbosa-Sampaio, Helena Cristina; Institute of Biology, State University of Campinas, Structural and Functional Biology
Amaral, Maria; Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, FHO/UNIARARAS |
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Involvement of M1 and M3 receptors in isolated pancreatic islets function during weight cycling in ovariectomized rats

Kayo Augusto Salandin Pacher¹, Thaís Furtado Camargo¹, Thiago Antonio Moretti Andrade¹, Helena Cristina Lima Barbosa-Sampaio², Maria Esméria Corezola do Amaral¹

¹Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, FHO/UNIARARAS, Araras, SP, Brazil

²Department of Structural and Functional Biology, Institute of Biology, State University of Campinas, SP, Brazil

**Corresponding author:**

Maria Esméria Corezola do Amaral

Graduate Program in Biomedical Sciences, Centro Universitário Hermínio Ometto, UNIARARAS, Av. Maximiliano Barutto nº 500, Jardim Universitário, 13607-339, Araras, SP, Brazil Tel.: +55 (19) 3543 1474; fax: +55 (19) 3543 1412

Tel.: +55 (19) 3543 1474; fax: +55 (19) 3543 1412

E-mail: esmeria@fho.edu.br
**Introduction**

Hypoestrogenism is a gonadal and reproductive dysfunction in women that is often associated with conditions such as obesity, insulin resistance, and diabetes (Chalvon-Demersayet et al. 2017). The control of food consumption can be effective in reducing body weight and retarding the progression of diabetes and hypertension. However, attempts to lose weight are often unsuccessful and can result in periods of weight loss followed by weight gain, in a phenomenon known as weight cycling (Bosy-Westphal et al. 2015).

There is a high prevalence of weight cycling in hypoestrogenic women, and its metabolic features are poorly understood. Studies involving humans and rodents have produced conflicting results, some showing benefits of weight cycling, while others have reported tissue inflammation and obesity (Pi-Sunyer et al. 2007; Villareal et al. 2006; Silva et al. 2012; List et al. 2012). Structural and functional adaptations in the endocrine pancreas have been observed in the presence of weight gain and changes in diet. Veras et al. (2014) found that ovariectomy in rats reduced the release of insulin by isolated islets stimulated with glucose, and that the function could be restored by administration of DHEA (dehydroepiandrosterone hormone). Estrogen protects pancreatic cells against glucolipotoxicity, oxidative stress, and apoptosis (Nadal et al. 2009). Studies that have demonstrated a correlation between estradiol and pancreatic β-cell function suggest that estrogens (estradiol) protect pancreatic β-cells function and survival from oxidative injury in female. Plasma estradiol levels in females may be endangered from insulin deficiency by inhibiting oxidative stress (Yokomizo et al., 2014). The susceptibility of pancreatic islets to oxidative stress is credited to their low level of expression of antioxidant enzymes (Lenzen et al. 1996) such as SOD1 (Cappelli et al. 2018), SOD2 (superoxide dismutase 1,2) and catalase (Takahashi et al. 2014). The genetic association of SOD2 with type II diabetes has been described (Miao et al 2009). Thus, the adaptation of the endocrine pancreas on ovariectomized rats can affect glucose and insulin concentrations by hypoestrogenism. This condition may cause islets oxidative stress (Kang et al. 2014). In this setting, the role of antioxidant enzymes is critical. Studies using humans and animals have shown that estrogen not only increases insulin sensitivity, but also preserves insulin production in the diabetic state (Tiano et al. 2015). In a study of the effects of estrogen therapy on pancreas islet function in postmenopausal women, over half of the individuals showed an increase in glucose-stimulated insulin secretion (Choi et al. 2005).
The regulation of glucose homeostasis relies on the coordinated function of β-cells (insulin-producing) and α-cells (glucagon-producing), which are stimulated according to the level of glucose, as well as the action of glucagon and insulin in peripheral tissues. Studies have shown that acetylcholine (ACh), which is the main neurotransmitter in peripheral parasympathetic nerves, acts to strongly stimulate insulin release (Gautam et al. 2006; Van Der Zee 1992; Gilon 2001). Studies using isolated islets derived from M3 muscarinic receptor knockout mice have shown the importance of this receptor for insulin secretion (Gautam et al. 2010; Zawalich et al. 2004).

The focus of the work reported here was on the use of a weight cycling model consisting of periods of ad libitum diet and periods of caloric restriction (40% of the ad libitum diet) in hypoestrogenic animals. In rodents, restricted diets have been found to result in reduced serum insulin, as well as glucose-stimulated secretion of insulin by isolated islets (Amaral et al. 2011). Despite these changes, glucose levels remained normal, which was attributed to compensations involving peripheral insulin sensitivity.

In the present study, evaluation was made of structural and functional adaptations of the endocrine pancreas caused by weight cycling in ovariectomized animals. Analysis of the expression of muscarinic receptors M1 and M3 enabled better understanding of the function of pancreatic islets in managing glucose homeostasis according to nutritional conditions.

**Materials and Methods**

**Animals**

The study was approved by the Animal Use Ethics Committee (CEUA) of Uniararas (protocol number 001/2015). The experiments employed 24 female Wistar rats aged two months, with average weight of 210±10 g. The animals were kept in individual cages, with temperature of 22±1 °C, 12 h light/dark cycles, and ad libitum water. Ovariectomy surgery was performed after anesthesia using a mixture of ketamine (100 mg/kg) and xylazine (10mg/kg), administered intraperitoneally. Trichotomy was performed in the ventral region between the costal margin and the thigh. After the incision and access to the abdominal cavity, the ovaries were located and submitted to ligature and section. Suture was performed with cotton thread (numbers 3 and 4), eliminating the dead space. Control animals underwent simulated surgery, using the same procedure. In this case, the ovaries were externalized, in order to induce similar stress, but were not ligatured and the reproductive apparatus was preserved.
The animals were placed in individual cages, divided into three groups (n=8 for each group): ShamAL (control; AL = ad libitum), OVXAL (ovariectomized), and OVXcycle (ovariectomized and submitted to weight cycling). The ShamAL and OVXAL groups received ad libitum commercial feed during the experimental period, while the OVXcycle group received 21 days of ad libitum commercial feed and 21 days of a caloric-restriction diet equivalent to 40% of the feed consumed by the OVXAL group. The caloric restriction (CR) was calculated on a daily basis, considering the weight of feed offered to the ad libitum animals and the amount of feed remaining, hence obtaining the amount of food ingested. One week before euthanasia, the ip.GTT (intraperitoneal glucose tolerance test) and ip.ITT (intraperitoneal insulin tolerance test) assays were performed in vivo. Euthanasia was performed by deep anesthesia using an overdose (3-fold the usual dose) of ketamine and xylazine. Blood was collected by cardiac puncture (n=8), the bilateral perirenal adipose tissue was removed and weighed (n=8), pancreatic islets were collected for Western Blot and insulin secretion analyses (n=3), and the pancreas was retrieved for immunohistochemical and histological analyses (n=5).

**Ip.GTT (intraperitoneal glucose tolerance test)**

The ip.GTT tests were performed after fasting the animals for 8 h (n=4 animals per group). The animals were administered glucose (2g/kg body weight) intraperitoneally. Blood samples were collected from the tail, before glucose overload (time zero) and at 30, 60, 90, and 120 min after glucose infusion. The blood glucose was determined using reagent strips and a glucose meter (Abbott, Chicago, USA).

**Ip.ITT (intraperitoneal insulin tolerance test)**

The ip.ITT tests were performed after fasting the animals for 6 h. The animals were administered standard crystalline insulin (0.75 U per kg body weight) intraperitoneally. Blood was collected at time zero (before insulin injection) and at times of 5, 10, 15, 20, 25, and 30 min. (McGuinness et al. 2009; Ayala et al. 2010) Blood glucose was determined using reagent strips and the Abbott glucose meter. The glucose disappearance constant (Kitt) was calculated using the formula: ln2/t_{1/2}. The serum glucose t_{1/2} value was calculated from the slope of the minimum regression curve, considering the linear phase of decrease of the plasma glucose concentration.
(Bonora et al. 1989). The blood glucose was determined using reagent strips and a glucose meter (Abbott, Chicago, USA).

Biochemical assays

Protein and insulin assays were performed with cardiac blood centrifuged to obtain serum. The protein analyze was performed using commercial kit, according to the manufacturer’s instructions (Laborclin, Pinhais, Paraná, Brazil) and insulin determination by ELISA, following the manufacturer’s instructions (Biotec Center, SPI-BIO and Ellipse Pharmaceuticals, Orléans, France).

Isolation of islets of Langerhans

The islets were obtained by the collagenase digestion method (Boschero et al. 1990). Laparotomy was performed and the common bile duct was cannulated in the liver-duodenum direction. After occlusion of the duct in the section adjacent to the duodenum, the pancreas was treated with infusion of 10 mL of Hanks solution containing collagenase at 0.8 mg/mL. The pancreas was then removed from the animal, transferred to a 25 mL test tube, and incubated for 18 min at 37 °C. At the end of the incubation, the tube was shaken manually for 1 min. After digestion, the material was washed 4 times with Hanks solution (without collagenase) and the islets were collected one by one using a previously silanized and stretched Pasteur pipette, under a magnifying glass.

Static insulin secretion

A group of 4 isolated islets was pre-incubated in Krebs bicarbonate solution for 45 min at 37 °C and pH 7.4, with 5.6 mM glucose. The pre-incubation medium was then discarded and the islets were incubated for a further period of 60 min in Krebs bicarbonate solution containing different concentrations of glucose (2.8, 8.3, and 16.7 mM) and carbachol (10 μM) with 8.3mM of glucose. After incubation, an aliquot of the supernatant was stored at -20 °C for subsequent insulin determination by ELISA, following the manufacturer’s instructions (Biotec Center, SPI-BIO and Ellipse Pharmaceuticals, Orléans, France).

Immunoblotting (Western Blot)
The isolated islets were homogenized for 30 s in protein extraction buffer, followed by centrifugation of the extract for 45 min at 12,000 rpm and 4 °C, in order to remove insoluble material. Aliquots of the supernatant were treated with Laemmli buffer containing 10 mM DDT and 50 μg portions were used for SDS-PAGE (6 to 10% Tris acrylamide), employing a minigel system (Miniprotein). After the run, the proteins were transferred to a PVDF membrane and incubated for 2 h in blocking solution, in order to reduce nonspecific protein binding. The membrane was then incubated overnight with specific antibodies for the different proteins: anti-β-actin (sc-477778), anti-SOD1 (sc-271014), anti-SOD2 (sc-137254), anti-catalase (sc-34284), anti-M1 (sc-7470), and anti-M3 (sc-9180), diluted 1:200 (Santa Cruz, California, USA). The membrane was then incubated for 2 h with specific antibodies of the chemiluminescent kit (Clarity Western ECL substrate, Bio-Rad), followed by examination using the Syngene G:BOX photodocumentation system. The band intensities were evaluated by densitometry, using Image J software.

**Pancreas histology**

The pancreas tissues were soaked in 10% formalin for 24h at ambient temperature, followed by dehydration, diaphanization, and embedding in Paraplast, according to the standard procedure for dehydration in alcohol. Each pancreas was cut into sections 5 μm thick, as follows: sections 1, 3, and 5 were collected, followed by discarding 20 sections, and subsequent collection until the entire organ had been used. The selected sections were used for immunohistochemical analysis of insulin and glucagon, Perls’ blue staining for iron deposition (revealed by the presence of blue ferric pigment), hematoxylin and eosin staining (with nuclei and cytoplasm stained purple and pink, respectively). The slides were observed and documented at 400x magnification using a Leica DM2000 photomicroscope software (version 4.1®). From each animal were obtained the diameters of all the isles totally delimited in the image, as an analysis tool was used the software Image-pro Plus (version 4.5.0.29). The estimated total beta-cell mass (mg) was calculated by multiplying the total (Σ) beta-cell area by pancreas weight and divided per pancreas section (Inuwa and Mardi 2005; d. Oliveira R.B. et al 2014). Qualitative histological evaluations for Perls were performed by two observers (KASP and TFC) considering presence and absence of labeling.
Insulin and glucagon immunohistochemistry

The localization of insulin employed the indirect immunoperoxidase method, using pancreas sections embedded in Paraplast. After blocking the nonspecific sites with endogenous peroxidase, using an ImmunoCruz kit (Santa Cruz Biotechnology, California, USA), the sections were incubated overnight, at 4 °C, with specific anti-insulin and anti-glucagon primary antibodies (Dako Carpinteria, California, USA). The sections were then washed and incubated with the specific secondary antibody. After washing and revealing the antigen-antibody complex formed with 10% diaminobenzidine (Sigma-Aldrich, Missouri, USA), 0.05 M TBS, and 0.2% hydrogen peroxide, the sections were stained with Ehrlich’s hematoxylin. They were then dehydrated, diaphanized, and mounted in synthetic balsam.

Statistical analysis

Comparative analysis of the results for the different groups was performed using ANOVA followed by Tukey’s post-test. The results were expressed as mean ± standard error of the mean (X±SEM), and the level of significance adopted was 5% (p<0.05).

Results

Metabolic characterization of the animals and analysis of the endocrine pancreas

The body weights of the different animal groups are shown in Figure 1. The animals of the OVXAL group showed significant increases of weight until the end of the experiment, indicative of hyperphagia. The OVXcycle group showed weight regain between the 70th and 91st days that was similar to the weight gain of the ShamAL animals, followed by weight reduction between the 91st and 112th days, due to the restricted diet, compared to the OVXAL and ShamAL animals.

Fasting glycemia (mg/dL) was lower for the OVXcycle group (76.5 ± 1.2), compared to groups OVXAL (128.7 ± 6.7) and ShamAL (95 ± 4.4) (Table 1). All the groups showed similar patterns for insulinemia (ng/mL) and total protein (g/dL). Peripheral adipose tissue (g) was lower for the OVXcycle animals (4.4 ± 0.7), compared to the OVXAL group (8.2 ± 0.8), but similar to that of the ShamAL group (5 ± 0.8). The pancreas weights were similar for all the groups (Table 1).
The values obtained for the intraperitoneal glucose tolerance test (ip.GTT) (Figure 2) and the area under the curve were statistically the same for all the groups. The values for the intraperitoneal insulin tolerance test (ip.ITT) and the glucose disappearance rate (Kitt) were higher for the OVXcycle group, compared to the OVXAL group.

**Insulin secretion, protein expression of muscarinic receptors, and analysis of antioxidant defense proteins in isolated pancreatic islets**

Higher secretion of insulin (ng/islet.60 min) induced by carbachol (µM) was found for the OVXcycle animals (5 ± 0.5), compared to groups ShamAL (2.5 ± 0.3) and OVXAL (3 ± 0.3) (Figure 3A). Insulin secretion by the isolated islets stimulated by 2.8 and 8.3 mM glucose was similar for all the groups. Under highly stimulatory glucose conditions (16.7 mM), insulin secretion was lower for the OVXAL and OVXcycle groups, compared to the ShamAL group (Figure 3B). Protein expressions of receptors M1 (Figure 3C) and M3 (Figure 3D) (arbitrary units) were higher (p<0.05) for OVXcycle (198 ± 13; 112 ± 26), compared to ShamAL (44 ± 3; 61 ± 6) and OVXAL (25 ± 2; 49 ± 4). Protein expressions of SOD1 (Figure 4A) and SOD2 (Figure 4B) (arbitrary units) were higher for OVXcycle (26150 ± 2092; 27163 ± 1441), compared to ShamAL (12247 ± 610; 21265 ± 1570) and OVXAL (8343 ± 1048; 17856 ± 1396). Catalase expression was similar for all the groups (Figure 4C).

**Pancreas histology and immunohistochemical analysis of insulin and glucagon**

No differences between the groups were found for the histological organization of the islets, as observed using HE staining (images not shown). The Perls’ blue staining results were indicative of higher iron deposits in the pancreas tissues of the OVXAL animals, compared to those in the ShamAL and OVXcycle groups. The results obtained for the immunohistochemical analyses of insulin and glucagon were as expected, with higher β-cell mass (mg) for groups OVXAL (6.2 ± 0.45) and OVXcycle (6.0 ± 0.39), compared to ShamAL (2.8 ± 0.22). The number of islets for section was similar for all the groups (Figure 5).

**Discussion**

The results for the animals subjected to weight cycling suggested that there was adaptation of the pancreatic islets to the imposed diet, as indicated by the enhancements
of the muscarinic response and the antioxidant defenses. This was reflected in insulin sensitivity, with lower secretion of insulin stimulated by glucose. The islets of the OVXAL animals showed loss of function, as evidenced by the failure of insulin secretion stimulated by high concentrations of glucose, together with iron deposition and deficiency of the antioxidant mechanisms, which was reflected in impaired insulin sensitivity. The deposition of iron in the pancreatic islets and lowered antioxidant defenses observed for the OVXAL group could have contributed to the lower M3 receptor expression, with consequent loss of the insulin secretion capacity of the β-cells. These features could be explained by a lack of estrogen, as reported elsewhere (Nadal et al. 2009; Veras et al. 2014). In contrast, the OVXcycle group showed adaptation to the weight cycling, with preservation of islet morphology and higher M3 receptor expression and insulin sensitivity (compared to the OVXAL group), even with lower insulin secretion.

This model of reduced insulin secretion was in agreement with the insulin pattern reported elsewhere for animals submitted to 40% caloric restriction for 21 days (Amaral et al. 2011). Previous studies have indicated that M1 and M3 receptors are predominant in pancreatic islets and modulate insulin secretion (Persson-Sjögren et al. 2001, 2000; Ahrén 2000; Iismaa et al. 2000; Miguel et al. 2002). In human islets, endogenous Ach stimulates insulin secretion via M3 and M5 receptors, as well as the secretion of somatostatin from δ-cells by means of M1 receptors. Somatostatin inhibits insulin secretion and therefore regulates β-cell function, characterizing the paracrine hormonal regulation pathway in pancreatic islets (Molina et al. 2014). The results for the OVXcycle animals were coherent with this hormonal regulation between the M1 and M3 receptors. Adaptive increase in muscarinic M1 and M3 receptor subtypes functional to regulate insulin and somatostatin secretion and thus glucose homeostasis suggesting a paracrine regulation in the pancreatic islets. Besides this, the increase of M1 and M3 receptors associated with a reduction in insulin levels could reflect response to the pancreatic adaptive response to the development of increased sensitivity to insulin peripherally to maintain glucose homeostasis induced by weight cycling ovariectomized (OVCcycle). Furthermore, it has been reported previously that ob/ob obese mice showed increased parasympathetic activity (Tassava et al. 1992), hyperinsulinemia, and insulin resistance (Edvell 1999). The islets of ob/ob rats release more insulin in response to glucose (Zawalich 1996) and acetylcholine (Tassava et al. 1992; Chen 1997), compared to the islets of lean mice (Chen 1997). It has been shown...
that the β-cells of obese mice attempt to compensate for the increased insulin requirement, but are unable to maintain normal maturation of insulin granules and their regulated release (Puff et al. 2011; Alarcon et al. 2016). It is possible that the lack of estrogen could provide a possible explanation for the differences in the patterns of insulin secretion found in the present work and in previous published studies, since the OVXAL animals were also obese. Furthermore, the expression of antioxidant enzymes in pancreatic β-cells is very low. High expressions of GPx, CAT, and SOD have been observed in the islets of diabetic rats (Lacraz et al. 2009). However, these islets are less sensitive to oxidative stress, showing that positive regulation of antioxidant defense is an intrinsic part of the mechanisms of self-protection against hyperglycemia (Lacraz et al. 2009). Thirty years ago, in vivo studies using rodents demonstrated that administration of SOD attenuated streptozotocin-induced diabetes (Gandy et al. 1982; Robbins et al. 1980; Asplund et al. 1984). Consequently, overexpression of Cu/ZnSOD reduces cytotoxicity induced by nitric oxide (NO) (Moriscot et al. 2000) and attenuates diabetes induced by alloxan (Kubisch et al. 1997). Increased resistance to oxidative stress has also been demonstrated in insulin-secreting cell lines and in islets from diabetic rats with overexpression of GPx or CAT (Harmon et al. 2009; Moriscot et al. 2003).

Overall, the findings of previous studies provide support for the expression of SOD1 and SOD2 in isolated islets found in the present work. It is likely that iron deposition in the islets of the OVXAL animals led to insulin insufficiency in the β-cells, due to oxidative stress. The same conclusions were reached by Delghingaro-Augusto et al. (2014). It is well known that iron is toxic towards the pancreas islets (Cooksey et al., 2010; Simcox et al. 2013; McClain et al. 2006) and can contribute to the pathogenesis of type 2 diabetes (Simcox et al. 2013). In epidemiological studies, increased rates of diabetes have been found in individuals with elevated levels of serum ferritin (Forouhi et al. 2007; Sun et al. 2013). Protective effects on pancreatic islet function in obese ob/ob mice have been observed with iron-restricted diets and iron chelation (Cooksey et al. 2010). Finally, the results of this work suggest that ovariectomy can accelerate the decline of pancreatic islet function, while weight cycling, involving receptors M1 and M3, was able to restore the characteristics of the islets.

**Conflict of Interest**

The authors declare that they have no conflict of interest.
Acknowledgments
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Author Contributions
KASP, TFC, TAMA, HCLBS, MECA conceived and designed research. KASP and TFC conducted experiments. MECA and TAMA wrote the manuscript. All authors read and approved the manuscript.

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**Legends**

**Figure 1.** (A) Body weights (g) of the animals in the ShamAL, OVXAL, and OVXCycle groups. (B) Timeline representation of animals groups. The values are expressed as mean ± standard error. Timeline legend: ShamAL mean 112 days of ad libitum commercial feed; OVXAL mean 112 days of ad libitum commercial feed; 28dAL mean 28 days of ad libitum commercial feed, 21dAL mean 21 days of ad libitum commercial feed and 21dCR mean 21 days of a caloric-restriction diet equivalent to 40% of the feed consumed by the OVXAL group. Different letters indicate statistically significant differences between the groups (p<0.05).

**Figure 2.** Intraperitoneal glucose tolerance test (ip.GTT) and corresponding areas under the curves, and intraperitoneal insulin tolerance test (ip.ITT) and glucose disappearance constants, for animals of the ShamAL, OVXAL, and OVXCycle groups. The values are expressed as mean ± standard error of the mean (X±SEM). Different letters indicate statistically significant differences between the groups (p<0.05).

**Figure 3.** Insulin secretion by isolated islets stimulated by carbachol (10 μM) with 8.3mM of glucose insulin secretion by isolated islets stimulated by different
concentrations of glucose, and expression of muscarinic receptors M1 and M3 (arbitrary
units) in isolated islets from the animals of groups ShamAL, OVXAL, and OVXCycle.
The values are expressed as mean ± standard error of the mean (X±SEM) and; n=12
wells with 4 islets each, for each animal group, for insulin secretion. Different letters
indicate statistically significant differences between the groups (p<0.05).

**Figure 4.** Expressions of SOD1 (A), SOD2 (B), and catalase (C) proteins (arbitrary
units) in isolated islets from the animals of groups ShamAL, OVXAL, and OVXCycle.
The values are expressed as mean ± standard error of the mean (X±SEM). Different
letters indicate statistically significant differences between the groups (p<0.05).

**Figure 5.** Representative photomicrographs of islets in sections of the pancreas tissues
of animals in groups ShamAL, OVXAL, and OVXCycle, at 400x magnification, stained
with HE and Perls’ blue (the arrows in the Perls’ blue images indicate granules with
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below representative of β cell mass and n˚islets/section from the animals of groups
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of the mean (X±SEM); n= 160-220 islets. Different letters indicate statistically
significant differences between the groups (p<0.05).
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Table 1. Fasting glycemia and insulinemia, total proteins, weight of the bilateral perirenal adipose tissue, and morphometric analysis of pancreas of ShamAL, OVXAL, and OVXcycle groups. The values are expressed as mean ± standard error of the mean (X±SEM; n=8). Different letters indicate statistically significant differences between the groups (p<0.05).

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<td>Glycemia (mg/dL)</td>
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<td>Pancreas weight (g)</td>
<td>0.76±0.078</td>
<td>0.66±0.09</td>
<td>0.64±0.06</td>
</tr>
<tr>
<td>Islet area (µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>9,704.53±570.22</td>
<td>12,547.66±663.63</td>
<td>10,670.44±1399.54</td>
</tr>
<tr>
<td>Beta-cell area (µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>7872.72±384.31</td>
<td>9179.23±469.77</td>
<td>8431.81±784.77</td>
</tr>
<tr>
<td>Alpha-cell area (µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1831.81±314.54</td>
<td>3368.43±664.75</td>
<td>2238.63±628.18</td>
</tr>
</tbody>
</table>

Data are means ± SEM.