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Abstract
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Reference

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Glucagon receptor antibody completely suppresses type 1 diabetes phenotype without insulin by disrupting a novel diabetogenic pathway

May-Yun Wang1, Hai Yan2,3, Zhiqing Shi2,3, Matthew R. Evans4, Xinxin Yu3, Young Lee3, Shuhwei Chen5, Annie Williams6, Jacques Philippe9, Michael G. Roth3,4, and Roger H. Unger2,3,1

1Touchstone Diabetes Center, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390; 2REMD Biotherapeutics, Inc., Camarillo, CA 93012; 3Beijing Cosci-REMD Biotherapeutics Inc., Beijing 102206, China; 4Department of Biochemistry and *Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390; 5University of Notre Dame, Notre Dame, IN 46556; 6University of Geneva School of Medicine, 1211 Geneva, Switzerland; and 7Department of Medical Service, Veteran's Administration North Texas Health Care System, Dallas, TX 75216

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Insulin monotherapy can neither maintain normoglycemia in type 1 diabetes (T1D) nor prevent the long-term damage indicated by elevated glycation products in blood, such as glycated hemoglobin (HbA1c). Here we find that hyperglycemia, when unaccompanied by an acute increase in insulin, enhances itself by paradoxically stimulating hyperglucagonemia. Raising glucose from 5 to 25 mM without insulin enhanced glucagon secretion ~two- to fivefold in InR1-G9α cells and ~18-fold in perfused pancreata from insulin-deficient rats with T1D. Mice with T1D receiving insulin treatment paradoxically exhibited threefold higher plasma glucagon during hyperglycemic surges than during normoglycemic intervals. Blockade of glucagon action with mAb Ac, a glucagon receptor (GCGR) antagonizing antibody, maintained glucose below 100 mg/dl and HbA1c levels below 4% in insulin-deficient mice with T1D. In rodents with T1D, hyperglycemia stimulates glucagon secretion, up-regulating phosphoenolpyruvate carboxykinase and enhancing hyperglycemia. GCGR antagonism in mice with T1D normalizes glucose and HbA1c, even without insulin.

glucagon receptor | antibody | type 1 diabetes | insulin

Nineteen years of insulin treatment in patients with type 1 diabetes (T1D) have made it clear that insulin alone cannot normalize glucose homeostasis or glycated hemoglobin (HbA1c) levels. Even optimally controlled patients may exhibit postprandial surges of glucose levels to three or four times normal (1, 2), which may explain why HbA1c levels below 6% are so rare in patients with T1D. Current thinking attributes these spikes in peripheral plasma glucose to insufficient uptake of incoming dietary glucose by peripheral target tissues as a result of a lack of insulin. As a consequence, they are often managed by a preprandial bolus of insulin and restriction of dietary carbohydrate. This strategy results in chronic iatrogenic hyperinsulinemia (3) in patients with “well-controlled” T1D and is responsible for a high incidence of hypoglycemic events, which can be life-threatening. In nondiabetic subjects, a glucose load suppresses glucagon levels by stimulating an acute transient rise in paracrine insulin from β-cells juxtaposed to the glucagon-producing α cells (4–6). This glucagon suppression converts the liver from an organ of glucose production to an organ of glucose storage (7). In T1D, paracrine insulin is lacking and is replaced by peripherally injected insulin. The resulting intrasulset insulin concentrations are but a small fraction of the paracrine concentrations of undiluted insulin that suppress glucagon in nondiabetic subjects (8, 9). In 1974, it was reported that hyperglycemia paradoxically stimulates glucagon secretion in dogs with chemically induced diabetes (10). More recently, plasma glucagon concentrations were reported to rise, with a tripling of hepatic glucose production, in normal rats continuously infused with glucose at a constant rate (11). Thus, there is evidence that in the absence of adequate insulin, elevated glucose might stimulate glucagon production, which in turn aggravates hyperglycemia. In this setting, the liver would not be reprogrammed to store incoming glucose but, rather, would continue to produce glucose as if it were still in the unfed state (12). This may play a major role in postprandial hyperglycemia (10).

Here we find that in T1D, hyperglycemia stimulates, rather than suppresses, glucagon secretion. This suggests that in T1D, a positive hormonal feedback loop enhances hyperglycemia by adding endogenously produced glucose to diet-derived glucose. If this is an important factor in the hyperglycemic surges that plague patients with T1D, then suppressing glucagon secretion or blocking glucagon action should eliminate the surges of hyperglycemia observed in T1D in mice.

To measure the normal response of pancreatic islets to elevated glucose, pancreata were isolated from normal mice and perfused with 5 or 25 mM glucose. Glucagon concentrations were measured in the perfusate. Raising the glucose concentration fivefold decreased glucagon concentration in the perfusate approximately sixfold (Fig. 1A). To determine the effect of increased glucose concentration on glucagon secretion without paracrine insulin, we measured glucagon levels in the medium of cultured InR1-G9α cells in 5, 10, and 25 mM glucose. The rise from 5 to 10 mM glucose caused an approximately threefold increase in glucagon secretion, and the rise from 10 to 25 mM caused another twofold rise (Fig. 1B). Because cultured cells may

Significance

Subcutaneous injections of insulin can only cure T1D patients with T1D but do not prevent hyperglycemic and hypoglycemic swings or decrease hemoglobin A1c levels to normal amounts. In mice treated with insulin alone, repeated episodes of transient elevated blood glucose cause long-term damage. We show that in mice with type 1 diabetes treated with insulin, the transient high blood glucose levels require production of glucagon, a hormone that will cause the liver to produce more glucose. Blocking the action of glucagon with an antibody to the glucagon receptor completely normalizes blood glucose and hemoglobin A1c in the complete absence of insulin therapy. Suppressing glucagon action in combination with low-dose insulin would be a superior treatment for type 1 diabetes.


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1To whom correspondence may be addressed. Email: roger.unger@utsouthwestern.edu or michael.roth@utsouthwestern.edu.
not reflect the behavior of native α cells in situ in T1D, we isolated pancreata from streptozotocin-induced insulin-deficient T1D rats and perfused them with 5, 10, and 25 mM glucose concentrations. When the perfusate glucose was increased from 5 to 10 mM, glucagon secretion increased fourfold (Fig. 1C). An increase in glucose from 10 to 25 mM increased glucagon secretion another fourfold. Insulin concentrations in all of these perfusates were below the detection limit of a radioimmune assay (RIA) (EMD Millipore). Previously, we reported that the streptozotocin treatment protocol we used resulted in the destruction of 93.4% of β cells (13). The fact that elevations of glucose stimulated glucagon secretion in the absence of an acute paracrine insulin release suggested that in animals with T1D, any rise in glucose would stimulate glucagon secretion and give rise to a cycle of self-enhancing hyperglycemia (3, 14). To investigate the possibility of such a diabetogenic pathway, we compared plasma glucagon levels in insulin-treated NOD/ShiLtJ T1D mice during and between hyperglycemic surges (Fig. 1D). The mice were treated with 0.1 U Levenir twice daily, and blood glucose was measured in the morning 17 h after an insulin injection (high blood glucose) and in the afternoon 7 h after an insulin injection (low blood glucose) (Fig. 1E). In mice receiving this insulin regimen, glucagon averaged $138 \pm 41$ pg/mL and insulin $3.9 \pm 1.1$ ng/mL in samples in which glucose averaged 500 ± 37 mg/dL. This glucagon concentration was significantly higher ($P < 0.05$) than the mean glucagon level of $55 \pm 35$ pg/mL measured in samples from the same mice when their glucose levels averaged $130 \pm 71$ mg/dL and insulin averaged $14.3 \pm 4.5$ ng/mL. These findings are consistent with a glucagon-mediated contribution to the surges of hyperglycemia.

To assess directly the effect on the liver of the hyperglucagonemia accompanying hyperglycemia in the absence of
endogenous insulin, we compared activation of key markers of glucagon action in liver. The phosphorylation of cAMP response element binding protein (CREB), a transducer of the glucagon signal, and the expression of a gluconeogenic glucagon target, phosphoenolpyruvate carboxykinase (PEPCK), were measured in T1D and nondiabetic mice. Compared with nondiabetic liver, there was a 3.5-fold elevation in phosphorylated CREB and a 2.5-fold increase in PEPCK expression in T1D livers (Fig. 2A–C). To demonstrate that these differences were glucagon-mediated, we treated T1D mice with a single injection of 5 mg/kg mAb B (15), a fully human, antiglucagon receptor (GCGR) antibody drug candidate under development by REMD Biotherapeutics, Inc. (15–17).

In mice treated with the monoclonal antibody mAb B, daily 10:00 AM blood glucose measurements averaged 85 ± 5 mg/dL and remained normoglycemic for 8 d (Fig. 2D), at which time livers were harvested. In the mAb B-treated T1D livers, phosphorylated CREB protein was reduced to nondiabetic levels and PEPCK protein expression was reduced below that of nondiabetic mice (Fig. 2A–C). Thus, the activation of hepatic gluconeogenesis in T1D mice was a result of their hyperglucagonemia and disappeared when glucagon actions were blocked.

If self-enhancing action of hyperglycemia is mediated by glucose-stimulated increase in glucagon in T1D, it follows that suppressing glucagon secretion should eliminate or reduce the problem. To test this, we placed T1D mice on a low dose of insulin (0.01 U twice daily) and then began continuous s.c. infusions of four peptides known to suppress glucagon directly or indirectly (18–22). A fifth, nonpeptidic suppressor (23), GABA, was given mixed in the chow. Each of the five reagents lowered glucagon levels, and in each case, this was accompanied by reduction of hyperglycemia from >600 mg/dL to 160 ± 75 mg/dL (P < 0.001; Fig. 3A). The average insulin concentrations in these samples were not correlated with either glucagon or glucose concentrations. With the exception of leptin, which reduced food intake by 50% compared with diabetic animals receiving insulin monotherapy, none of these glucagon suppressors caused a significant reduction in food intake.

If the glucose-lowering effects of glucagon suppressors resulted entirely from reduced glucagon secretion, rather than from off-target actions, therapy with a GCGR antibody should cause as dramatic an improvement as the glucagon-suppressing agents. Mice with chemically (streptozotocin)-induced T1D and a starting hyperglycemia of 325 ± 72 mg/dL were injected i.p. once each week with 7.5 mg/kg anti-GCGR antibody mAb Ac (15) (Fig. 3B), and blood glucose was measured weekly for 12 wk. Blood glucose concentrations returned to normal (~90 mg/dL) in the mice treated with the antibody 1 wk after a single dose (first time point), and this normalization continued for the duration of treatment. Body weight did not change significantly between the control and antibody-treated groups of mice from the start to finish of the experiment. In the vehicle-treated control mice, blood glucose levels rose to 540 ± 70 mg/dL during the 12-wk study. At the end of this treatment, HbA1c was measured as an indication of chronic hyperglycemia. In the mice treated with mAb Ac, HbA1c levels were normal (4 ± 1%), whereas in the control mice, HbA1c averaged 11 ± 1% (Fig. 3C). GLP-1 averaged 3.64 ± 0.9 pmol/L in the control mice and 3.63 ± 0.52 pmol/L in the mice treated with antibody. By immunohistochemistry, the ratio of insulin-positive cells to glucagon-positive cells in islets observed in sections of pancreas taken from five control mice at the end of the study was 0.15, but using cells from five antibody treated mice, the ratio was 0.16. Because the ratio of β cells to α cells in wild-type mice is ~6.0 (24), the ratios observed are those expected for severe ablation of β cells by streptozotocin. The fact that the ratio did not change between the two groups of animals indicates that the antibody treatment did not induce α-cell hyperplasia in this experiment.

Discussion

The present report provides the first evidence to our knowledge that acquired ablation, similar to congenital ablation of glucagon action, completely prevents the hyperglycemia and elevated HbA1c of T1D. Our work further supports the essential role of
glucagon in the pathophysiology of diabetes, as was first proposed in 1975 (25). Subsequently, it was supported by demonstrations that diabetes was ameliorated by every glucagon suppressor used in every species tested. This included human T1D (26). Similarly, glucagon receptor antibodies have been shown to lower hyperglycemia in T2D (15), but this is the first demonstration, to our knowledge, of normalization of glycemia and HbA1c in complete insulin deficiency, a result that, to our knowledge, has never been achieved with existing therapies. Previous neutralization of glucagon itself reduced, but did not normalize, blood glucose levels (27–29). Synthetic glucagon receptor antagonists (30–33) similarly improved the diabetes but did not normalize it.

Despite the abundance of scientific support for glucagon’s central role in the disease, plus the calculation by Cahill that without glucagon the liver was incapable of producing hyperglycemia, the idea has persisted that insufficient insulin action, rather than excess glucagon action, causes diabetes. However, during the 9 decades of insulin therapy, it has become evident that correcting the deficit in insulin action cannot restore glucose homeostasis or HbA1c levels to normal, despite the hyperinsulinemia it induces. Incontrovertible evidence of glucagon essentiality to diabetes was recently reported in congenital glucagon receptor-null mice in which total insulin deficiency failed to raise blood glucose or glucose tolerance above normal (13). Now, in the present study, we show in genetically normal rodents that acquired loss of glucagon action caused by glucagon receptor immunoneutralization also prevents and reverses T1 diabetes.

This study also proposes a novel self-enhancing pathophysiologic pathway to severe hyperglycemia. In 1971, it was reported that without glucagon action, the liver cannot produce enough glucose to sustain hyperglycemia in insulin-deficient T1D mice. Here we present evidence for progressive enhancement of hyperglycemia by glucose-stimulated glucagon secretion that occurs when a blood glucose rise is unaccompanied by a physiologic rise in paracrine insulin. The anti-GCGR mAb Ac maintained normoglycemia and normalized HbA1c in severely insulin-deficient mice with impending diabetic ketoacidosis. Most remarkably, A1c normalization was achieved without inducing iatrogenic hyperinsulinemia (3), which may result in excess cardiovascular mortality (34). The antibody without any concomitant insulin administration completely prevented the T1D phenotype.

Thus, the metabolic phenotype of T1D can be eradicated by blocking glucagon action on the liver. The fact that the foregoing results were obtained in rodents raises the question of species differences from humans. Earlier work from our laboratory showed in human T1D that suppression of glucagon secretion with somatostatin eliminated postprandial hyperglycemic surges (26). This is evidence that the concept of antagonizing glucagon action as an effective therapy for diabetes will apply to humans. In any event, the approach of antagonizing the glucagon receptor for therapy in diabetes will need to be validated in human clinical studies.

Taken together, this suggests that insulin replacement therapy alone, without glucagon suppression by an agent other than insulin, is an incomplete strategy for regulating T1D. A more rational approach would be to first lower the unphysiologically high insulin doses now in common use (3) to doses that do not produce peripheral hyperinsulinemia and hypoglycemia. This lowering of insulin would increase glucagon and blood glucose levels, but both could be reduced by a glucagon-suppressing agent or by an anti-GCGR antibody, such as mAb B and mAb Ac.

**Methods**

**Effect of Glucose on Glucagon Secretion by InR1-G9 Cells.** For the experiments shown in Fig. 1, InR1-G9 (35, 36) cells, obtained from J.P., were cultured at 37 °C in a humidified 5% (vol/vol) CO2 incubator in DMEM (high glucose) media, containing 10% (vol/vol) FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, and 1 mmol/L sodium pyruvate, without insulin. Cells harvested from cultures lower than passage 25 were used. Cells were seeded into a 384-well plate to a density of 10,000 cells per well in a volume of 30 μL. After a 2 h incubation to allow the cells to attach to the plate, the medium was replaced with the desired concentration (5, 10, or 25 mmol/L) of glucose, and the cells were grown for 20 h at 37 °C. Five μL of a 1:1,000 dilution (in PBS) of a rabbit antiglucagon antibody was added to each well and incubated for 30 min. Next, 5 μL of a 20 μg/mL (2 μg/mL final concentration) of AlphaLisa protein A conjugated acceptor beads (PerkinElmer) was added to each well and allowed to incubate for an additional 30 min. After incubation,
a 1 mmol/L final concentration of biotinylated-glucagon (Anaspec; catalog number 60274-1) was added to each well and incubated for 60 min. Last, streptavidin-conjugated AlphaLISA donor beads were added to a final concentration of 8 μg beads/mL to give a final sample volume of 50 μL. The mixture was incubated at room temperature for 2 h. All additions and incubations were made in subdued lighting conditions according to the manufacturer’s instructions. The assay was measured on an Envision plate reader (PerkinElmer), and triplicate values were averaged.

Perfusion of Pancreata. To determine the effects of high glucose on glucagon secretion by intact a cells in islets of rodents with T1D, six lean wild-type Zucker rats were treated twice with 100 mg streptozotocin 1 wk apart to induce severe T1D. Insulin in blood collected from animals treated with two doses of streptozotocin was undetectable by RIA. Seven days after the second dose of streptozotocin, they were fasted for 17 h and then anesthetized with 60 mg/kg body weight of sodium pentobarbital administered intraperitoneally. The pancreas was isolated and perfused in situ at 37 °C. The perfusate contained 1× KRB buffer (129 mmol/L NaCl, 4.8 mmol/L KCl, 0.2 mmol/L CaCl2, 1 mmol/L KH2PO4, 1 mmol/L MgSO4, 5 mmol/L NaHCO3) plus D-glucose at 5, 10, or 25 mmol/L concentration. Perfusates were supplemented with 0.5% (wt/vol) bovine albumin fraction V and gassed for 30 min with 95% (vol/vol) O2 and 5% CO2 for 30 min. The perfusate flow rate was maintained at 4 mL/min. After a 10-min equilibration period with 5 mmol/L D-glucose, insulin was increased to 10 mmol/L for 10 min and to 25 mmol/L for another 10 min. The temperature was kept constant at 37 °C. The effluent samples were collected from the portal vein, without recycling, into prechilled tubes containing 25 mmol/L EDTA and aprotinin, and frozen after −20 °C for later use. The concentrations of glucagon and insulin were measured by RIA (catalog numbers GL-32K and RI-13K; TED Millipore), according to the manufacturer’s instructions. For measurements on normal rodents, pancreata from C57BL/6N mice from Charles River Laboratories International were perfused by the protocol described here for rats.

Glucagon Suppression in Vivo in Mice with T1D. Eight-week-old NOD/ShiLtJ female mice from the Jackson Laboratory that had developed diabetes were assigned to one of three groups: insulin monotherapy (0.1 U twice daily at 10:00 AM and 5:00 PM), reduced insulin monotherapy (0.01 U twice daily at 10:00 AM and 5:00 PM), and reduced insulin therapy plus a glucagon suppressor. Mimosomatic pumps (ALZET model 2011; ALZET Omotic Pumps) were implanted between the scapulae under ketamine/xylazine anesthesia (0.1 mL per 20 g body weight). The pumps delivered agents at a rate of 1 μL/h during a 7-d period and were replaced every 7 d. They were loaded with various known or suspected glucagon suppressors, including recombinant leptin at 20 mg/mL (Amylin Pharmaceuticals, Inc.), pramlintide acetate at 1 g/mL (Amylin Pharmaceuticals, Inc.), and glucagon-suppressing antibodies at 5 mg/mL liraglutide (Novo Nordisk AS), 250 mg/mL exenatide (Amylin Pharmaceuticals, Inc.). An untreated control group received PBS delivered by Alzet pump, plus twice daily s.c. injections of 0.01 U of the long-acting insulin analog (Leverim U-100; Novo Nordisk) administered at 10:00 AM and 5:00 AM. An additional group of mice received 0.01 U Leverim injected twice daily plus the orally effective (23) glucagon suppressor GABA (catalog number A-2129; Sigma) mixed with the powdered food (4% [wt/vol] fat). Food intake, body weight, and blood glucose were monitored daily. Blood samples were obtained at 10:00 AM, 1:00 PM, and 5:00 PM. Insulin was administered twice daily at 10:00 AM and 5:00 PM, after obtaining the blood glucose specimen. For all animal experiments, principles of laboratory animal care (NII publication no. 85–23, revised 1985) were followed according to protocols reviewed and approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Treatment of NOD Mice with Monoclonal Antiglucagon Receptor Antibodies. The two monoclonal antibodies used differ by only one amino acid not involved in glucagon binding and have equivalent affinities for glucagon (15). Diabetic NOD mice were injected s.c. with buffer (control, n = 5) or buffer containing 5 mg/kg mAb B monoclonal antibody to the GCCR (n = 6), and blood glucose concentration was monitored daily for 8 d. Male Balb/C mice at 8–10 wk of age were injected IP with 60 mg/kg streptozotocin daily for 5 consecutive days, and after 14 d, were injected with vehicle (control) or 7.5 mg/kg mAb Ac (n = 10) weekly for 12 wk. Fasting blood glucose levels were measured weekly. After 12 wk, blood HgA1c was measured in fresh whole blood with a Toshiba TBA-40FR auto-analyzer.

Plasma Measurements. Blood samples (100 μL) were collected in tubes containing 0.15 T/1mL aprotinin and 10 mmol/L EDTA. Samples were centrifuged, and aliquots of plasma were frozen at −20 °C. Plasma glucagon was measured using a rodent glucagon RIA kit (Linco Research). Mouse plasma insulin was measured by an ELISA kit (Crystal Chem, Inc.).

Immunoblotting Analysis. Total protein extracts prepared from liver tissues of NOD mice with or without the treatment of glucagon suppressors were resolved by SDS/PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The blotted membrane was blocked in 1× Tris-buffered saline (TBS) containing 0.1% Tween and 5% (wt/vol) nonfat dry milk (TBST-MKL) for 1 h at room temperature with gentle, constant agitation. After incubation with primary antibodies anti-phospho-CREB, anti-CREB, or anti-PEPCK (Cell Signaling Technologies), or anti-γ-tubulin (Sigma) in freshly prepared TBST-MKL for 4 h, the membranes were washed two times with TBST buffer. This was followed by incubating with secondary anti-rabbit or mouse horseradish peroxidase-conjugated Ig anti-bodies in TBST-MKL for 1 h at room temperature with agitation. The membranes were then washed three times with TBST buffer, and the proteins of interest on immunoblots were detected by an ECL plus Western blotting detection system (GE Healthcare Life Sciences). The corresponding bands were quantified using NIH Image software (version 1.6, available at rsb.info.nih.gov/nih-image).

Histochecmy. The pancreas was removed from five control and five test animals at the end of the experiment shown in Fig. 3B and fixed in 4% (vol/vol) formaldehyde overnight, then embedded in paraffin, and each entire pancreas was sectioned. Paraffin was removed from the sections with xylen, and the sections were rehydrated through a graded series of alcohol in water. Sections were treated with 3% (vol/vol) H2O2 for 10 min to block endogenous peroxidase activity, and in 10% (vol/vol) goat serum for 30 min to block nonspecific antibody binding. Sections were then incubated sequentially with rabbit anti-glucagon antibody (Abcam, ab18461) and biotinylated goat anti-rabbit IgG (Abcam, ab97049) and detected with ABC-HRP Kit (Vector Laboratories, pk-4100) and DAB peroxidase substrate kit (Vector Laboratories, sk-4100), according to the manufacturer’s instructions. Sections were then incubated with guinea pig antiinsulin antibody (Abcam, ab7842) and biotinylated goat anti-guinea pig IgG (Abcam, ab6907), and the antibody was detected with ABC-HRP Kit (Vector Laboratories, sk-4000) and DAB peroxidase substrate kit (Vector Laboratories, sk-3100). Sections were counterstained with hematoxylin for 15 s, rinsed in water, and dehydrated through graded solutions from 70% (vol/vol) to 100% ethanol and then into 100% xylene. Sections were mounted in Permount (Fisher, SP15–100), dried, and observed by microscopy. Pancreatic islets in pancreas sections were captured at a magnification of 200×, and the positive labeled cells in all islets were evaluated. The total area containing insulin-positive and glucagon-positive cells (islet) was measured, as was the area of individual cells stained with each marker within each islet. Dividing the area of positive cells for insulin or glucagon by the total area per islet ×100 gives the percentage of each cell type in each islet.

Statistical Analysis. Results are presented as mean ± SEM and were evaluated by Student’s t test for two groups or by ANOVA. For histochecmy, one-way ANOVA testing (SSPS 17.0) was applied among the groups; P < 0.05 was accepted as significant.

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