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Galantamine Attenuates Type 1 Diabetes and Inhibits Anti-Insulin Antibodies in Nonobese Diabetic Mice

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Type 1 diabetes in mice is characterized by autoimmune destruction of insulin-producing pancreatic β-cells. Disease pathogenesis involves invasion of pancreatic islets by immune cells, including macrophages and T cells, and production of antibodies to self-antigens, including insulin. Activation of the inflammatory reflex, the neural circuit that inhibits inflammation, culminates on cholinergic receptor signals on immune cells to attenuate cytokine release and inhibit B-cell antibody production. Here, we show that galantamine, a centrally acting acetylcholinesterase inhibitor and an activator of the inflammatory reflex, attenuates murine experimental type 1 diabetes. Administration of galantamine to animals immunized with keyhole limpet hemocyanin (KLH) significantly suppressed splenocyte release of immunoglobulin G (IgG) and interleukin (IL)-4 and IL-6 during KLH challenge *ex vivo*. Administration of galantamine beginning at 1 month of age in nonobese diabetic (NOD) mice significantly delayed the onset of hyperglycemia, attenuated immune cell infiltration in pancreatic islets and decreased anti-insulin antibodies in serum. These observations indicate that galantamine attenuates experimental type 1 diabetes in mice and suggest that activation of the inflammatory reflex should be further studied as a potential therapeutic approach.
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INTRODUCTION

Type 1 diabetes is associated with substantially increased rates of morbidity and mortality, accounting for costs exceeding $14.9 billion in health care costs in the U.S. each year (1–11). It is characterized by decreased insulin secretion and severe hyperglycemia, which can lead to ketoacidosis, coma and death. The disease pathogenesis is attributed to immune-mediated destruction of the insulin-producing β-cells of pancreatic islets. Histopathological findings in type 1 diabetes includes inflammation and destruction of pancreatic islets with infiltration of macrophages, T cells and other immune cells (12–19). Titers of autoreactive antibodies are significantly increased in patients suffering from type 1 diabetes, including antibodies specific for insulin (IAA), glutamic acid decarboxylase (GAD), protein tyrosine phosphatase (ICA512 or IA2A) and zinc transporter protein (ZnT8) (20–23). There are currently no effective treatments for type 1 diabetes. Anti–B-cell antibodies (rituximab), anti-CD3 antibodies (otelixizumab and teplizumab) targeting T cells and the Diamyd vaccine (GAD immunotherapy) have all failed to meet endpoints in recent clinical trials (24–30).

Recent advances in understanding neural control of innate immunity reveal that neural reflexes, including the inflammatory reflex and the cholinergic antiinflammatory pathway, control cytokine release and inflammation (31–39). The cholinergic antiinflammatory pathway is defined as a vagus nerve signal that culminates on T-cell acetylcholine release and activation of α7 nicotinic acetylcholine receptor (α7nAChR) on splenic macrophages, which inhibits proinflammatory cytokine release (40–44). Nerve stimulation and administration of α7 receptor agonists are in clinical development for treatment of inflammatory diseases (39,45–47). Recent evidence also links activation of the cholinergic antiinflammatory pathway to a reduction in antibody production in spleen, specifically lower antibody titers and B-cell activity during *Streptococcus pneumoniae* infection (48). Here we reasoned that the cholinergic antiinflammatory pathway would attenuate inflammation and serum antibody titers in murine type 1 diabetes.

Galantamine, a centrally acting acetylcholinesterase (AChE) inhibitor clinically approved to treat Alzheimer's disease

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(49,50), is an activator of the cholinergic antiinflammatory pathway (35,51). We recently reported that galantamine treatment of mice with high fat diet–induced obesity significantly alleviates weigh gain, obesity-associated inflammation, hyperglycemia and insulin resistance (52). The nonobese diabetic (NOD) mouse is a model of type 1 diabetes, spontaneously developing antibodies against self-antigens, with islet infiltration starting at 3–4 wks, leading to later islet destruction and hyperglycemia around 16–18 wks (53). In addition, similar antibodies, as observed in humans, have been described in the nonobese diabetic (NOD) mouse model of type 1 diabetes (54–56). Accordingly, here we administered galantamine to NOD mice beginning at a preclinical stage and measured blood glucose and serum antibodies. We found that galantamine administration attenuates type 1 diabetes–associated hyperglycemia, confers protection against islet inflammation and decreases serum titers of diabetes-related autoimmune antibodies.

MATERIALS AND METHODS

Animals

NOD (4–5 wks old) and Balb/c (6–8 wks old) mice were obtained from The Jackson Laboratory. Food and water were available *ad libitum*. Mice were used in subsequent experiments after at least a 14-d adaptation period. All procedures were performed in accordance with the National Institutes of Health (NIH) guidelines (57) under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research.

Cytokine and Antibody Determination

Balb/c mice were injected intraperitoneally with 100 μg keyhole limpet hemocyanin (KLH) (Calbiochem) + 50% Imject Alum (Thermo Scientific) in 200 μL saline two times, 2 wks apart. Two weeks after the second injection, 4 mg/kg galantamine was injected intraperitoneally. Splenocytes were harvested 4 h later, and erythrocytes were lysed with red blood cell lysis buffer (Sigma-Aldrich) and cultured in RPMI, HEPES, penicillin/streptomycin, L-glutamine, 1% nonessential amino acids and β-mercaptoethanol. Cells were exposed to increasing concentrations of KLH for 48 h, and media were analyzed for cytokine and antibody content. Cytokines were measured by multiplex enzyme-linked immunosorbent assay (ELISA) (Quansys), and IgG antibodies were measured by ELISA. Briefly, plates were coated with 20 μg/mL KLH antigen in phosphate-buffered saline (PBS) (Life Technologies) overnight, followed by blocking with 1% bovine serum albumin (BSA) in PBS for 2 h. Plates were washed with PBS + 0.01% Tween-20 (PBST) three times. Blood collected by capillary tube from nicked mouse tail was spun at 5,000*g*, and then serum was extracted and diluted 1:1,000 to 1:10,000 in PBS + BSA. A total of 100 μL diluted serum was incubated on coated plates for 1 h at room temperature and was then washed three times with PBST. A 1:2,000 dilution of horseradish peroxidase–conjugated sheep anti-mouse IgG (GE Healthcare) was added to the plates for 1 h at room temperature and was then washed again. Plates were then developed using OptEIA TMB substrate (BD Biosciences), and optical density at 450 nm was measured. Total IgE levels were determined by ELISA with rat anti-mouse IgE (BD Biosciences) coated plates, probed with biotinylated rat anti-mouse IgE antibody, followed by horseradish peroxidase–conjugated streptavidin.

Drug Administration

Five-week-old female NOD/ShiLtJ mice were injected intraperitoneally with 1 mg/kg galantamine (Calbiochem) or vehicle control in 200 μL normal saline, daily, until the end of the experiment. Blood glucose was measured once weekly by using a Freestyle Freedom Lite meter (Abbott). Mice were deemed diabetic after 2 consecutive blood glucose readings >200 mg/dL. For therapeutic experiments, NOD/ShiLtJ mice with overt diabetes (blood glucose >200 mg/dL for 2 wks in a row, 16–22 wks of age) were injected intraperitoneally with 1 mg/kg galantamine or saline, daily, with monitored blood glucose. At the end of the experiment, mice were euthanized with CO_2, and pancreas, spleen and serum were collected and processed for further analyses.

Tissue Processing and Insulitis Scores

Mice were euthanized at 17 wks, and pancreas was fresh-frozen in optimal cutting temperature (O.C.T.) medium. Diabetic and nondiabetic mice were both included in the analysis of insulitis. The 5- to 7-μm slices were obtained by cryostat sectioning, mounted and fixed in acetone, allowed to dry at room temperature and stained with Mayer's hematoxylin for 5 min. Insulitis was scored by using a bright-field microscope in double-blinded manner (blinded to both treatment groups and diabetic state). At least 50 islets from at least three disparate sections of each mouse pancreas were then scored as follows: 0, no insulitis; 1, peri-insulitis; 2, moderate (<70%) insulitis; 3, complete (>70%) insulitis (Supplemental Figure S1).

Antibody Determination

Plates were coated with antigens including insulin (human insulin; Sigma), histone II-A (from calf thymus; Sigma), myelin basic protein (MBP) (from mouse; Sigma), myelin oligodendrocyte glycoprotein (MOG) (immunodominant epitope of mouse MOG; Sigma) and deoxyribonucleic acid (DNA) (from calf thymus; Sigma). IgG levels were determined by ELISA as previously described using plates coated with each respective antigen.

Statistics

Blood glucose, KLH antibody responses and cytokine responses were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni posttest. Diabetes-onset survival curves were analyzed by log-rank (Mantel-Cox). Islet scores were analyzed by the χ^2 test. All tests with a p value of <0.05 were considered statistically significant. Statistical analyses were performed by using Graphpad Prism 6 software. Unless oth-

erwise stated, all numbers are given as average ± standard error of the mean.

All supplementary materials are available online at www.molmed.org.

RESULTS

Galantamine Attenuates Antibody Release by Splenocytes

We first examined the effect of galantamine administration on splenocyte antibody release. Mice were immunized intraperitoneally with keyhole limpet hemocyanin in alum twice, 2 wks apart. Fourteen days after the second immunization, mice were injected with galantamine (4 mg/kg) or saline intraperitoneally 4 h before euthanasia, and isolated splenocytes were cultured in the presence of KLH *in vitro*. Splenocytes from mice administered galantamine produced less KLH-specific IgG, while producing more total IgE (Figure 1). In addition, galantamine administration resulted in reduced interleukin (IL)-4 and IL-6, but not IL-2, release by splenocytes (Figure 2). No changes in cell numbers were observed.

Galantamine Reduces Serum Levels of Anti-Insulin Antibodies in NOD Mice

NOD mice have elevated levels of autoantibodies (55). To investigate the effect of galantamine on serum antibody levels, titers of anti-insulin, anti-histone, anti-DNA, anti-MOG and anti-MBP antibodies were measured in galantamine-administered and control NOD mice. IgG antibodies specific to insulin were significantly reduced in galantamine-administered animals (Figure 3A), whereas antibodies directed against histone, DNA, MOG and MBP were unaffected (Figures 3B–E). These results indicate a selective suppressive effect of galantamine administration on insulin-specific antibody production in this model.

Galantamine Prevents Hyperglycemia and Diabetes Onset

To study the preventative effects of galantamine in type 1 diabetes, 4- to

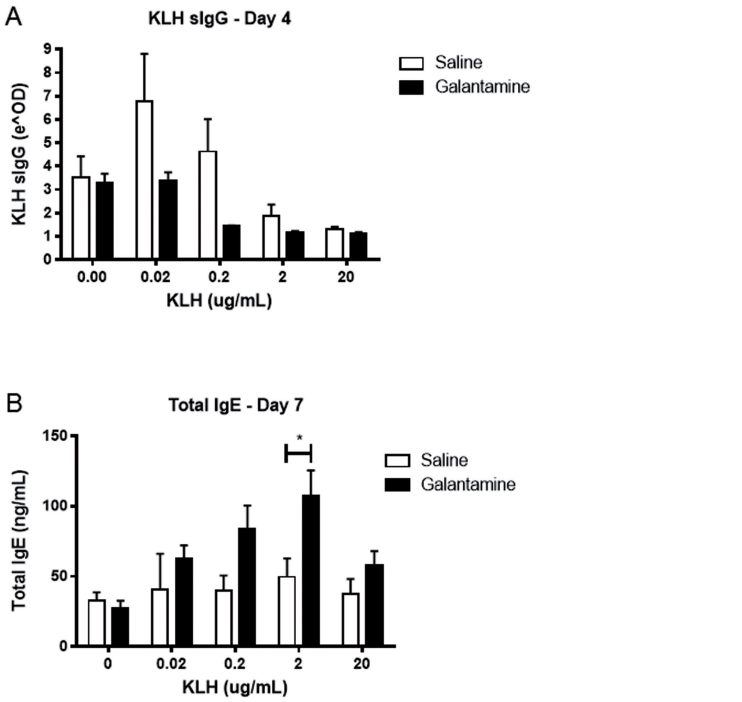

Figure 1. Galantamine alters antibody responses in immunized mice. KLH immunized mice were administered galantamine (4 mg/kg) or saline 4 h before death. Splenocytes were extracted, plated at 2 × 10^5 in 96-well plates and incubated with indicated μg/well KLH. KLH-specific IgG (A) and total IgE (B) was determined by ELISA. Galantamine affected antibody levels (IgG, $p < 0.05$; IgE, $p < 0.01$ by two-way ANOVA; *$p < 0.05$ by Bonferroni posttest).

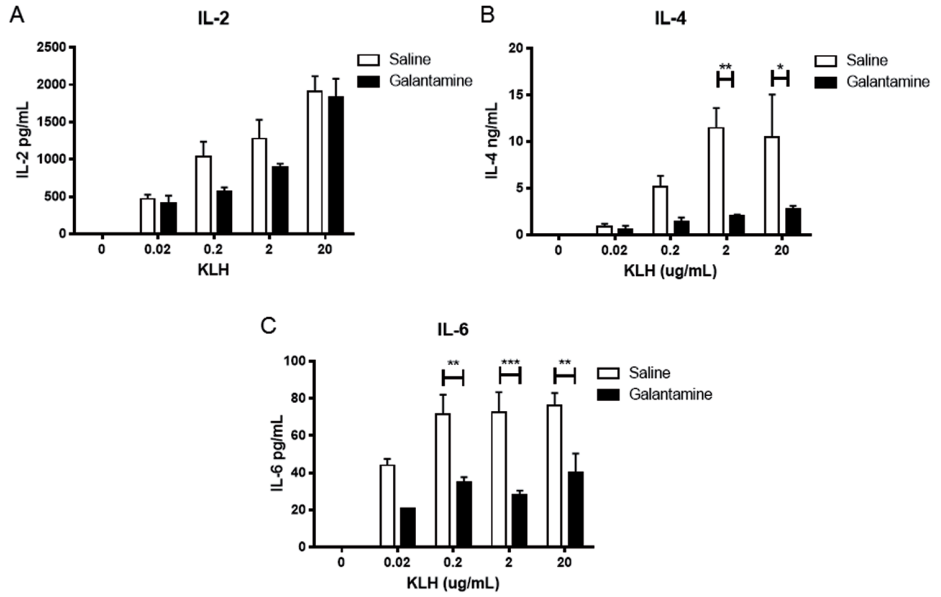

Figure 2. Galantamine alters cytokine responses in immunized mice. KLH immunized mice were administered galantamine (4 mg/kg) or saline 4 h before death. Splenocytes were extracted, plated at 2 × 10^5 in 96-well plates and incubated with indicated μg/well KLH. Cytokines were determined on d 7 by Quansys multiplex ELISA. (A) Galantamine did not significantly affect IL-2. (B) IL-4 ($p < 0.001$) and IL-6 ($p < 0.0001$) were significantly different in galantamine-treated animals (two-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by Bonferroni posttest).

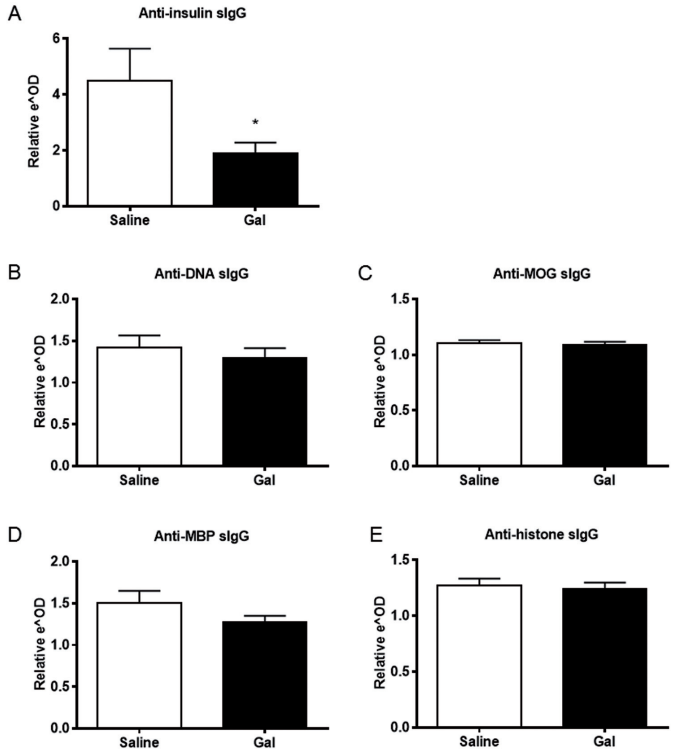

Figure 3. Galantamine administration reduces levels of circulating pathogenic anti-insulin antibodies. Serum from galantamine- or saline-administered was applied to ELISA plates coated with antigens and then probed with anti-mouse antibodies. Serum anti-insulin antibodies were reduced in animals administered galantamine (A) ($p > 0.05$), but other common NOD autoantibodies were not affected (B–E).

5-wk-old NOD mice were administered 1 mg/kg galantamine (n = 11) or saline (n = 10) intraperitoneally, 200 μL daily for 20 wks. Blood glucose was monitored weekly to determine the onset of hyperglycemia, defined as glucose >200 mg/dL. Mice administered galantamine did not develop hyperglycemia until wk 19, whereas almost all mice administered saline rapidly developed hyperglycemia by wk 14 (Figure 4A). The average glucose levels in saline-administered animals was 226.6 ± 60.3 mg/dL by wk 14; similar levels were not reached in galantamine-administered animals until wk 19 (227.8 ± 52.3 mg/dL) (Figure 4A). Additionally, the prevalence of diabetes (two consecutive blood glucose levels >200 mg/dL) in mice administered galantamine was lower (Figure 4B, $p < 0.05$). Galantamine-administered mice that did develop diabetes rapidly progressed to high levels of blood glucose (data not shown).

To determine the efficacy of galantamine in treating established diabetes, NOD mice with 2 consecutive weeks of blood glucose >200 mg/dL were administered 1 mg/kg galantamine daily. Galantamine treatment did not significantly alter blood glucose levels, compared with vehicle treatment (Supplementary Figure S1). Together, these results indicate that galantamine in the context of preclinical type 1 diabetes at relatively early stages of disease progression is efficacious in delaying onset of hyperglycemia.

Galantamine Attenuates Pancreatic Islet Inflammation

To determine to effect of galantamine on immune cell infiltration in pancreatic islets, pancreata from 17-wk-old female NOD mice administered 1 mg/kg galantamine or saline intraperitoneally daily were investigated by using histochemical methods. The 7-μm sections were obtained from throughout the pancreas, at least 100 μm apart. Insulitis was scored from 0 to 3, as indicated in Figure 5A. Administration of galantamine reduced the severity of insulitis (Figure 5B) and improved the overall insulitis score in galantamine-administered animals (Figure 5C, χ^2 $p < 0.0005$).

DISCUSSION

Here, we show that administration of the acetylcholinesterase inhibitor galantamine (35,51,58) delays elevation of blood glucose levels and onset of diabetes in the NOD model. In this disease model, galantamine reduces pancreatic islet inflammation and lowers disease-specific antibody levels without reducing levels of other tested IgG antibodies. Furthermore, splenocytes derived from galantamine-administered mice released less IL-4, IL-6 and IgG in response to antigen re-exposure *in vitro*.

The observation that splenocytes derived from galantamine-administered mice released less IgG and higher IgE levels in response to immunized antigen *in vitro* suggests that galantamine stimulates class-switching of B cells from IgG to IgE. In addition, the lower IL-4 and IL-6 secretion by splenocytes from galantamine-administered animals after antigen exposure suggests an environment less supportive of B-cell antibody release, including suppression of Th2 helper T cells. These findings highlight a previously unrecognized antiinflammatory effect of galantamine in preclinical type 1 diabetes.

Autoimmunity in NOD mice is initiated in the pancreatic lymph nodes, but not in the spleen, at 2–3 wks of age (59). Treatment starting from 5 wks of age could influence a mechanism that slows the progression of insulitis and ultimately the onset of clinical diabetes separate from the initial priming for autoimmunity to islet autoantigens. We examined cytokine content by ELISA from pancreatic samples at 12 wks of age

after 8 wks of galantamine administration. The lysate of these samples included embedded pancreatic lymph nodes. We found no difference in levels of interferon (IFN)-γ, IL-17, monocyte chemotactic protein 1 (MCP-1) or IL-1β (data not shown).

Changes in cellular populations could be an important component of galantamine's antiinflammatory effects. To examine changes in populations, we obtained single cell isolates from both spleen and total pancreas, including lymph nodes, taken from mice administered galantamine for 8 wks. We examined $CD3^+CD4^+$, $CD3^+CD4^+FoxP3^+$ and $CD3^+CD8^+$ populations by flow cytometry. We observed no difference in percentages of these populations in treated animals compared with vehicle control in both spleen and pancreas (data not shown). Fewer cells were observed in the pancreata of galantamine-treated animals (data not shown). It is plausible that galantamine may suppress the inflammatory activity of the immune cells, but does not alter the immune cell composition. The specific effect of galantamine on cellular function remains to be elucidated, and additional analyses would provide useful data.

Galantamine has previously been shown to exert antiinflammatory effects through brain-mediated and vagus nerve–dependent signaling (35,51). The vagus nerve innervates the pancreas, and vagus nerve cholinergic output controls pancreatic endocrine and exocrine secretion (60–62). A role for the vagus nerve has also been shown in suppressing pancreatic inflammation; surgical transection of the vagus nerve (vagotomy) results in exacerbated murine pancreatitis, thus indicating a tonic antiinflammatory role of these innervations (63). The vagus nerve also innervates the liver, and it is known that vagus nerve–mediated signaling suppresses hepatic glucose production (64–66), one of the main determinants of fasting blood glucose levels. We suggest that galantamine-mediated activation of the vagus nerve reduces pancreatic inflammation and delays onset of diabetes.

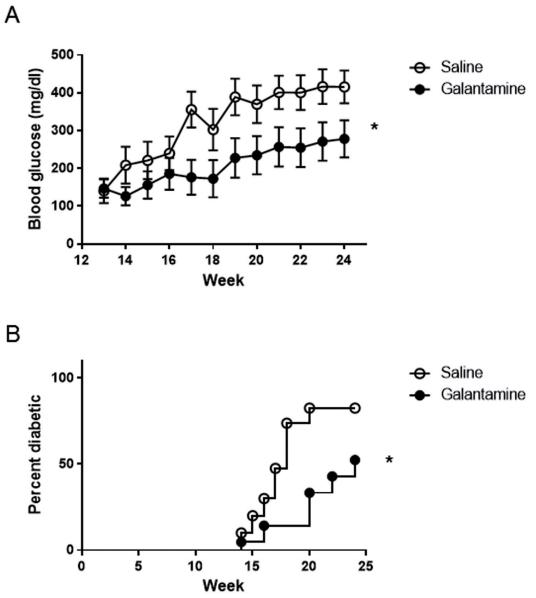

Figure 4. Galantamine delays the onset of hyperglycemia and diabetes. The 1 mg/kg galantamine (n = 11) or saline (n = 10) was administered intraperitoneally daily to NOD mice beginning at 5 wks of age. Blood glucose levels were measured weekly, with two successive weeks of blood glucose >199 mg/dL, indicating onset of diabetes. Administration of galantamine (A) significantly reduced the average blood glucose over time ($p < 0.05$, two-way ANOVA), and (B) significantly delayed onset of diabetes ($p < 0.05$, Mantel-Cox, results of two combined experiments).

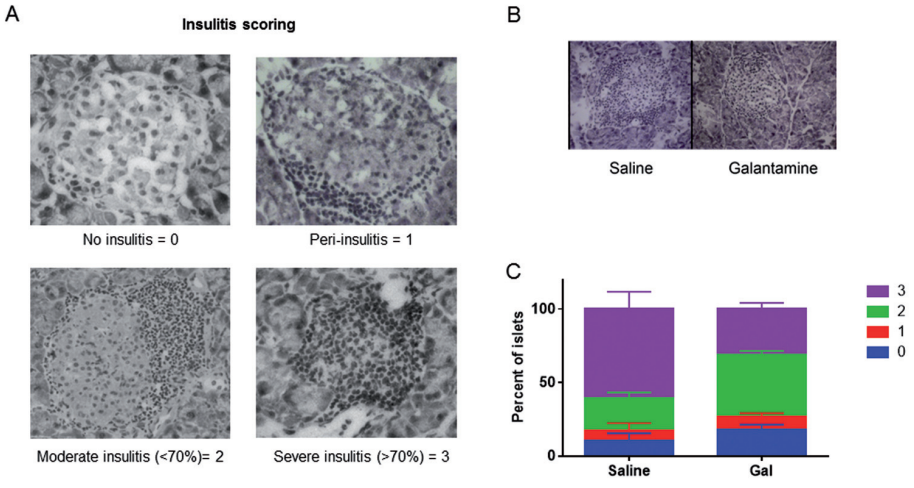

Figure 5. Daily administration of galantamine decreases islet infiltration by immune cells. (A) Insulitis scoring scheme examplars. Islets with no infiltrating mononuclear cells were scored as 0, peri-islet inflammation only = 1, moderate intra-islet inflammation occupying <70% of the islet = 2, severe-complete intra-islet inflammation occupying >70% of the islet = 3. (B, C) Pancreata from 16-wk-old NOD mice injected intraperitoneally with saline or galantamine from 5 wks of age were isolated and frozen in OCT media and then sliced at 10 μm. At least 50 total islets from three disparate areas of each pancreas were scored blindly. Administration of galantamine (B) reduced the severity of insulitis in NOD mice (representative images), as well as (C) improved the overall level of insulitis in the pancreas (n = 5, χ^2 $p < 0.0005$).

In this context, it is possible that metabolic effects of vagus nerve activation on hepatic glucose release may also play a role (67). Future studies should address the role of these and other mechanisms in mediating galantamine efficacy in type 1 diabetes.

CONCLUSION

Galantamine is in clinical use for treatment of Alzheimer's disease patients in the United States and has been used for decades in Europe in treating myasthenia gravis and Alzheimer's disease and in children with autism spectrum disorders (49,50,68). This abundant clinical experience in adult and pediatric populations should facilitate potential experimental use of galantamine in the treatment of type 1 diabetes. Other therapeutic approaches, including β-cell transplantation or β-cell regeneration have been explored (69–76). In light of our current findings, it would be interesting to further study whether galantamine treatment in combination with islet restoration would improve clinical outcomes in established type 1 diabetes.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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