Original Article

Involvement of γ-Aminobutyric Acid and N-methyl-D-aspartate Receptors in Diabetic Gastropathy in Rats: Possible Beneficial Effect of Prolonged Treatment with Insulin and Magnesium Supplement

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ABSTRACT

Gastrointestinal dysfunction is a severe and common complication in diabetic patients. Some evidence shows that gamma-aminobutyric acid (GABA) and glutamate contribute to diabetic gastrointestinal abnormalities. Therefore, we examined the impact of prolonged treatment with insulin and magnesium supplements on the expression pattern of GABA type A (GABA-A), GABA-B, and N-methyl-D-aspartate (NMDA) glutamate receptors as well as nitric oxide synthase 1 (NOS-1) in the stomach of type 2 diabetic rats. Twenty-four male Wistar rats were randomized to four groups (six rats each): 1) control, 2) type 2 diabetes: rats fed with a high-fat diet for three months + a low dose of streptozotocin (35 mg/kg), 3) type 2 diabetes + magnesium, and 4) type 2 diabetes + insulin. The expression of NOS-1, GABA-A, GABA-B, and NMDA receptors was detected using western blotting. The NOS-1 expression was substantially diminished ($P<0.01$), while the expression of GABA-A ($P<0.001$), GABA-B ($P<0.001$), and NMDA ($P<0.001$) receptors was enhanced in the stomach of diabetic rats relative to control. Treatment with magnesium and insulin improved NOS-1 expression in diabetic rats, although this effect was greater in magnesium treatment alone. Magnesium also restored the expression of GABA-A and GABA-B receptors in diabetic rats to control values. Moreover, insulin treatment improved GABA-A receptor expression in diabetic rats ($P<0.05$). No considerable alterations were detected in NMDA receptor levels in the treatment groups. The results suggest a significant role of magnesium and insulin in improving gastric motility and secretory disorders associated with diabetes through modifying the expression of GABAergic receptors.

Keywords: GABA Receptor, Magnesium Supplement, NMDA Receptor, NOS-1, Type 2 Diabetes

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1. Introduction

Gastrointestinal (GI) dysfunction (gastropathy) is a severe and common complication in diabetic patients that may develop in more than 75% of the individuals. The pathophysiological symptoms of diabetic gastropathy are complex and include delayed gastric emptying (gastroparesis), impaired gastric accommodation, nausea, visceral hypersensitivity, and gastric dysrhythmias (1). Gastroparesis is more common in patients with longer periods of diabetes, higher Hba1c levels, and frequent episodes of hyperglycemia (2). Previous research has indicated an irreversible decrease in neuronal nitric oxide synthase (nNOS) expression in the stomach of non-obese diabetic rats without a decrease in nitrergic neurons in diabetes (3). Nitrergic system defect is regarded as a principal factor in the pathogenesis of gastroparesis. Inhibition of nNOS can reduce NO synthesis, which in turn decreases smooth muscle relaxation (4). Assessment of the effects of different drugs on nNOS inhibition has revealed a potential role of nNOS in gastropathy treatment. In addition to nNOS, dysregulation of the GABAergic or glutamatergic signaling pathways is assumed to be implicated in motor-secretory disorders in gastropathy. The GABAergic neurons are found in the submucosa, more specifically in the myenteric plexus of the GI tract, with a specific recurrence in the large intestine (5). The GABA has been suggested to modulate both secretory and motor GI tract activities. It serves its impacts via three types of GABA receptors, including GABA-A, GABA-B, and GABA-C (6). Activation of ionotropic GABA-A and GABA-C receptors is usually associated with stimulation of cholinergic and noncholinergic/nonadrenergic neurons, resulting in contraction or relaxation of GI smooth muscle (7). In the stomach, endogenous GABA is often involved in the negative regulation of spontaneous mechanical activity through a GABA-A-dependent facilitator impact on noncholinergic/nonadrenergic neurotransmission (8). The GABA-B-dependent rigorous regulation of gastric cholinergic signaling has also been shown to contribute to GABA-related modulation of gastric acid secretion (9). Ionotropic glutamate receptors are ligand-gated ion channels that include three subtypes: NMDA (N-methyl-d-aspartate), amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainite. Gastric glutamatergic pathways are principally extrinsic in origin and may contribute to inhibitory or excitatory motor responses, depending on the stimulus and the area involved. Glutamate receptors in the gastric mucosa participate in the transmission of sensory information related to vagal afferent fibers and play a role in food digestion (5). It has been shown that all NMDA receptor subunits in the gastric wall are also present in neurons and mucosal cells (10). Pharmacological evidence indicates that NMDA and kainate receptors in myenteric neurons located in smooth muscles of the fundus of rats have a potent stimulatory effect (11). The NMDA receptor inhibition has been demonstrated to accelerate gastric emptying, which in turn can indirectly alter mechanoreceptor afferent signaling and lead to increased meal size (12). Magnesium deficiency has been linked to diabetes mellitus development. Similar to humans, a negative correlation is found between plasma magnesium and hyperglycemia in diabetic rats. The etiology of hypomagnesaemia in diabetes is complicated and is thought to contribute to diabetes-related complications (13). In addition, myocardial disorders associated with magnesium deficiency were found in animal models of diabetes, such as rats and rabbits. In addition, insulin resistance was associated with reduced concentration of magnesium in both plasma and erythrocytes (14). In another study, Solati et al. (2019) reported improvements in blood glucose, lipid profile, and blood pressure following dietary magnesium supplementation in diabetic patients (15). Thus, it is possible that a common mechanism involving magnesium is responsible for certain complications of diabetes. By reducing tyrosine kinase function of insulin receptors and intracellular calcium changes, hypomagnesaemia has been shown to reduce insulin function, which in turn may contribute to the development of diabetes. In the central nervous system, magnesium blocks voltage-dependent NMDA receptor channels, which are responsible for the abnormal processing of sensory information. In one study, magnesium supplementation attenuated chronic hypersensitivity and spinal cord NMDA receptor phosphorylation in a rat model of diabetic neuropathic pain (16). Therefore, by blocking NMDA receptors with magnesium, new treatment approaches can be developed to attenuate diabetes complications, including gastroparesis. Here, we investigated the therapeutic impact of magnesium supplements on the expression pattern of NOS-1 protein, as well as GABA-A, GABA-B, and NMDA receptors.

2. Materials and Methods

2.1. Study design

In this study, 24 male Wistar rats (120-130 gr, 4-week-old) were grouped into non-diabetic control and diabetic groups (n=6 for each). The rats were housed in a 12:12 light: dark and a temperature of 23 ± 2°C. After one week of acclimatization, six male rats were kept under a normal diet as a non-diabetic control group, and the rest of the rats were fed with a high-fat diet for three months according to Table 1. To induce type 2 diabetes, streptozotocin (STZ; 35 mg/Kg) was injected intraperitoneally confirmed by

the measurement of blood glucose in rats. To measure the blood sugar without causing any stress to the animal, a very small slit was created at the end of the tail and the sugar was measured using a glucometer. A blood sugar of more than 250 mg/dl was considered as successful type 2 diabetes induction. Diabetic animals were allocated to three subgroups: 1) diabetic rats, 2) diabetic rats treated with magnesium for two months, and 3) diabetic rats treated with insulin for two months. During the treatment course, the body weight and blood sugar of each rat were measured weekly. Finally, the animals (25–week-old) were sacrificed in a proper manner (overdose with ketamine-xylazine) and the stomachs were immediately removed for the evaluation of expression of NOS-1 protein, as well as GABA-A, GABA-B, and NMDA receptors and stored in liquid nitrogen following storage in a freezer with -70°C.

2.2. Western blot study

At the end of the experiments, anesthesia was induced with ketamine and xylazine. Lysis buffer with the following composition was used to lyse the tissues. After centrifugation at 12,000 cycles per minute for 10 minutes, the protein level in the supernatant was determined. Equal amounts of proteins were placed on a 10% polyacrylamide gel. After electrophoresis, the samples were transferred to polyvinylidene difluoride membranes with primary antibodies against NOS-1 (Santa Cruz Biotechnology, Inc), GABA-A receptor alpha 1 (abcam), GABA-B receptor (Santa Cruz Biotechnology, Inc) and NMDAε1 (E-4) (Santa Cruz Biotechnology, Inc). Subsequently, the samples were conjugated with appropriate HRP secondary antibodies and then visualized on X-ray films via enhanced chemiluminescence detection. The Western blot result was scanned and densitometric analysis was performed to quantify the bands using Image J software (National Institutes of Health, Bethesda, MD, USA). Protein levels were normalized to β-actin, which is used as an internal control protein.

Table 1. Components of high-fat diet

Amount (gr/Kg)	Ingredients
365	Powdered base ration
310	Lard
250	Casein
10	Cholesterol
65	Mixture of vitamin and
minerals	
3	Methionine
1	Yeast powder
1	Chloride sodium

2.3. Data analysis method

Analysis was conducted using Graph Pad Prism (version 9.0.0). Results are presented as Mean ± SEM. One-way ANOVA (analysis of variance) and Tukey's follow-up tests were used for data analysis. A $P<0.05$ was considered statistically significant.

3. Results

We found that NOS-1 receptor expression in the stomach of the diabetic rats was substantially declined ($P<0.01$; Figure 1) and treatment with insulin and magnesium improved the pathologic finding in the stomach. Although treatment with magnesium increased NOS-1 expression in the diabetes + magnesium group by 25% compared to the diabetic group ($P<0.05$), NOS-1 levels remained low in this group compared to the control ($P<0.05$; Figure 1). There was also a substantial enhancement of GABA-A receptor expression in the diabetic rats relative to the control group ($P<0.001$; Figure 2). A marked decrease was also observed in GABA-A receptor expression in diabetes + insulin and diabetes + magnesium groups compared with the diabetic group ($P<0.01$ and $P<0.001$, respectively; Figure 2). In addition to a significant reduction in the levels of GABA-A receptor expression in treated groups compared to the diabetic group, magnesium treatment caused a significant reduction in the protein content of GABA-A receptor in the diabetic rats compared to the insulin-treated group ($P<0.001$). GABA-B receptor expression was also significantly higher in the diabetic group relative to the control group ($P<0.001$; Figure 3). Compared with the diabetic group, GABA-B receptor expression was notably diminished in the diabetes + magnesium relative to the diabetic group ($P<0.05$; Figure 3). The NMDA receptor expression in diabetes, diabetes + insulin, and diabetes + magnesium groups was significantly higher than that of the control group, although compared to the diabetic group, a non-significant reduction in the expression of NMDA receptor was observed in the diabetic group + magnesium (Figure 4). The diabetic rats exhibited a higher level of fasting blood sugar than the control rats ($P<0.001$; Figure 5). Prolonged treatment with insulin or magnesium supplements notably attenuated the fasting blood sugar in the diabetic rats ($P<0.001$) relative to the untreated diabetic rats.

4. Discussion

The present study evaluated alterations in the expression of gastric GABA-A, GABA-B, and NMDA receptors as well as NOS-1 protein in diabetic rats treated with insulin and magnesium. Results showed a substantial decline in NOS-1 expression in the diabetic group relative to the other groups.

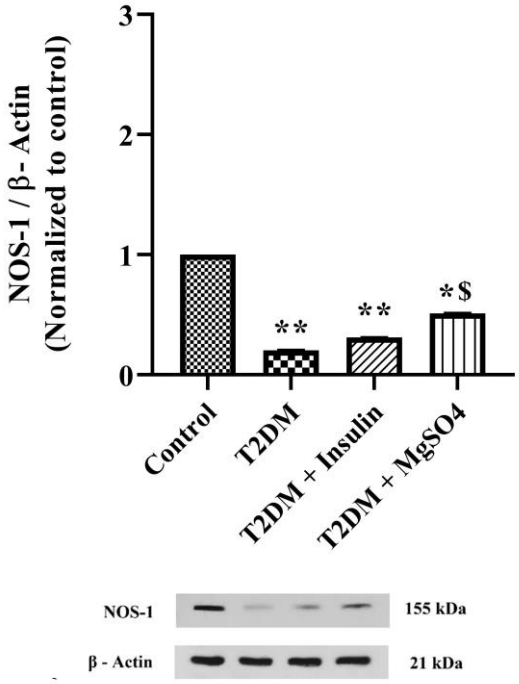

Figure 1. NOS-1 expression level in different groups compared to the control group. Compared to the control group, the expression level of NOS-1 in the diabetic group, diabetic group treated with insulin or magnesium was significantly declined.* p<0.05, ** p<0.01 indicate a significant difference vs control and $ p<0.05 vs the diabetic groups. T2DM: Type 2 diabetes mellitus

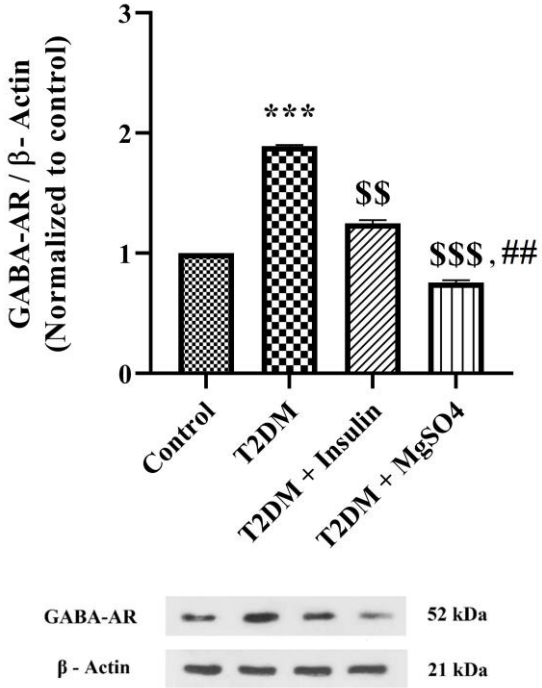

Figure 2. GABA-A receptor expression level in different groups compared to the control group. Compared to the control group ،the expression level of GABA-A receptor in the diabetic group is significantly higher; while its expression level decreases significantly in the diabetes + magnesium and insulin groups compared to the diabetic group with no treatment. *** p<0.001 Indicates a significant difference vs control, $$ p<0.01 and $$$ p<0.001 vs the diabetic groups and ## p<0.01 between treated groups. T2DM: Type 2 diabetes mellitus.

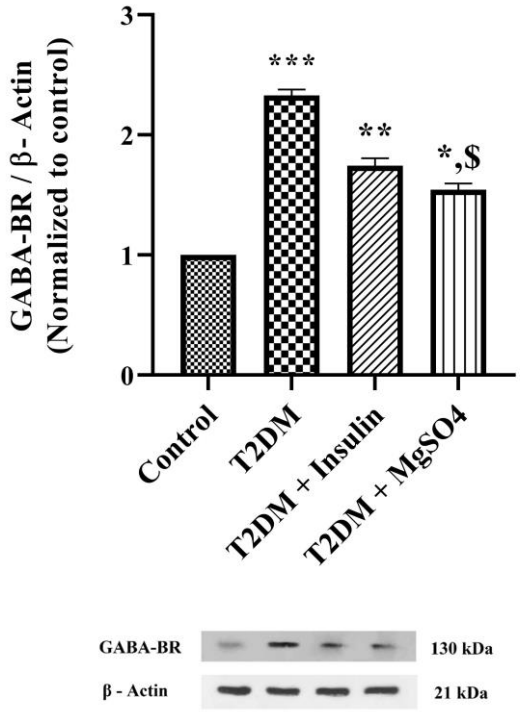

Figure 3. GABA-B receptor expression level in different groups compared to the control group. The expression level of GABA-B receptor showed a significant increase in the diabetes, diabetes + insulin and diabetes + magnesium groups compared to the control group. Although, diabetes + magnesium group showed a significant decrease in the expression level of GABA-B receptor compared to the diabetic group. $p<0.05$, ** $p<0.01$, *** $p<0.001$ indicate a significant difference vs control and $ $p<0.05$ vs the diabetic groups. T2DM: Type 2 diabetes mellitus

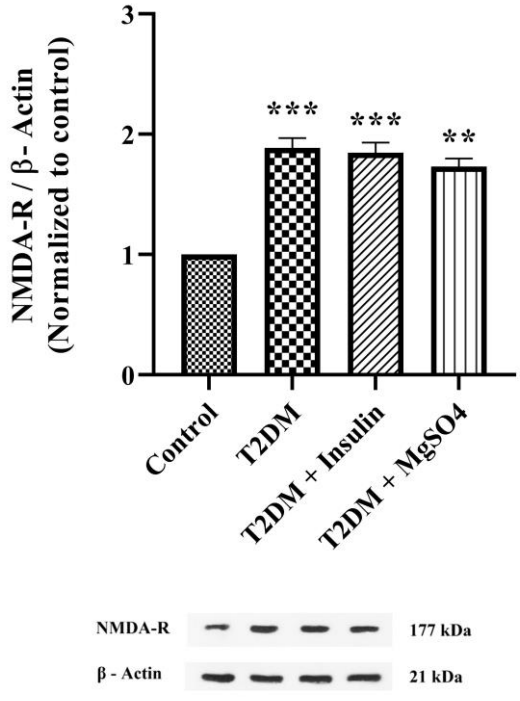

Figure 4. The expression level of NMDA receptor in different groups compared to the control group. The expression level of NMDA receptor showed a significant increase in the diabetic, the diabetes + insulin and diabetes + magnesium groups compared to the control group. *** $p<0.001$ indicates a significant difference vs control group. T2DM: Type 2 diabetes mellitus

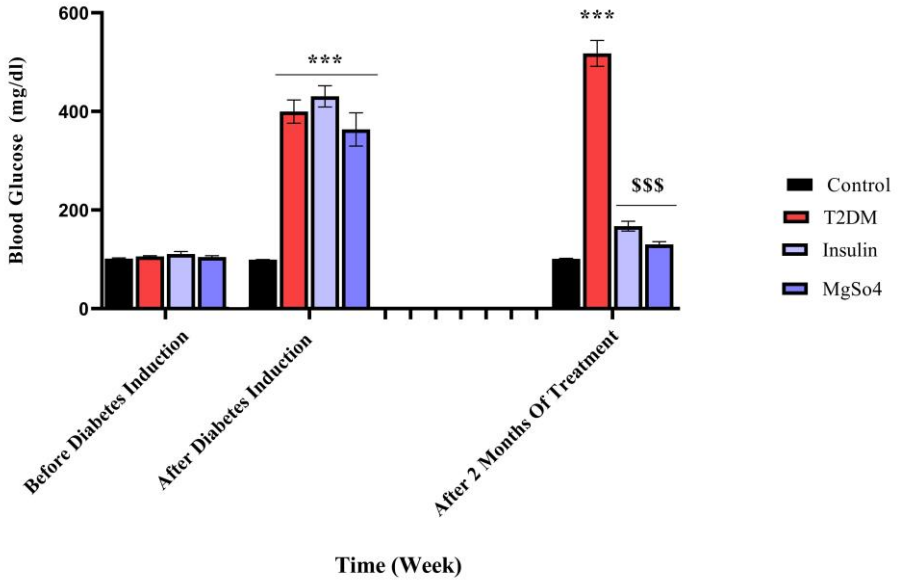

Figure 5: Effects of long term treatment with insulin or Mg2+ on fasting blood glucose concentrations in T2DM rats (n=6). Data are expressed as mean ± SEM, * * * p< 0.001 vs. control and $$$ p< 0.001 vs T2DM rats.

Treatment, especially with magnesium, could improve this pathologic feature in diabetic rats. Diminished nNOS expression appears to be one of the most common findings in animal and human studies evaluating diabetic gastropathy. In this regard, nitrergic abnormalities, including decreased nNOS-reactive cells in the gastric myenteric network, declined nNOS expression and impaired gastric relaxation mediated by nitrergic activity have been reported in animal models of diabetic gastropathy. Nitric oxide produced by nNOS acts as an inhibitory neurotransmitter and has been shown to improve the survival of interstitial cells of Cajal in the GI tract (17). In our study, the expression level of GABA-A and GABA-B receptors was considerably augmented in the diabetic rats relative to the controls. In peripheral tissues, GABA, an extracellular signaling molecule, has been shown to activate GABA-A and GABA-B receptors in the plasma membrane of cells. The GABA-A receptors are pentameric ion channels, while GABA-B receptors are G protein-coupled receptors. In recent years, studies have focused on GABA's role in modulating GI functions, including motility, secretion, and the intestinal immune system, through its possible effects on the enteric nervous system (ENS), which is a complex neural network located in the intestinal wall. The GABA receptors are highly expressed in multiple parts of the GI tract. The presence of GABA in submucosal nerve cells and mucosal nerve fibers allows it to regulate the transport of intestinal fluids and electrolytes in the GI tract (18). Stimulation of GABA-A and GABA-C receptors is usually associated with increased release of neurotransmitter from enteric neurons, leading to contraction or relaxation of GI smooth muscle (18). In contrast, GABA-B receptor activation is principally associated with presynaptic suppression of voltage-gated calcium channels, leading to reduced acetylcholine release from enteric neurons. In addition, some studies have reported possible effects of GABAergic pathways on gastric acid secretion (9). More research is required to confirm these results. Studies in rodents and humans have found that both GABA-A receptors and GABA-B receptors play an important role in β-cell replication and survival in normal and diabetes conditions (19). Moreover, GABA has been indicated to be beneficial to type 2 diabetes. In a study in high-fat diet-fed mice, oral treatment with GABA ameliorated glucose tolerance, blood glucose, and insulin sensitivity (20). This may be due to GABA inhibition of obesity-associated inflammation and upregulation of Treg responses, since GABA was found to inhibit insults and the production of inflammatory cytokine. These data suggest the role of GABA signaling in regulating cell survival and regeneration, and immune function. An enhancement of GABA receptor expression in the gastric enteric tissue in diabetes condition has been suggested to maintain GABA signaling for the survival of the gastric secretory and muscle cells. In our previous study, insulin and magnesium treatment was effective in improving the inflammatory condition of the stomach tissue (21), and this reduction of inflammation can reduce the need for enhanced GABA signaling. In addition, magnesium has been shown to regulate GABA receptor activity (22). It was also found to regulate glutamate levels by interacting with GABA receptors. In our study, treatment with magnesium and insulin altered the expression of GABA receptors and NOS-1 in the stomach of diabetic rats, which in turn may improve diabetes-induced GI complications in rats. The NMDA receptor is found in neurons and consists of GluN1, GluN2, and GluN3 subunits (23). The NMDA receptor blockade was found to accelerate gastric emptying, which can indirectly affect mechanoreceptor afferent signaling and lead to increased meal size. The GI motility is predominantly regulated by inputs from cholinergic neurons and NMDA receptors have been shown to be highly expressed in enteric cholinergic neurons. The motility response

of the GI tract is primarily determined by the activation of the myenteric network and a combination of external contractile and relaxant signals. The possible involvement of peripheral NMDA glutamate receptors in GI motility has attracted great interest, but which specific pathways and co-released neurotransmitters contribute to the modulation of GI motility mediated by glutamate NMDA receptors are not still understood (24). The NMDA receptors are assumed to modulate intestinal motility by inducing muscle contraction, neuroplasticity, or visceral hypersensitivity (10). Hence, pharmacological manipulation of internal and external glutamatergic pathways innervating the stomach might be useful in the treatment of gastric motility and secretory abnormalities, especially in diabetes conditions (12). Studies have demonstrated that plasma glutamate is enhanced in various chronic oxidative stress conditions, including diabetes, insulin resistance, obesity, and cancer (25), causing excitotoxicity and enhancing inflammatory responses. Furthermore, prolonged high level of glutamate was found to accelerate the onset of type 2 diabetes and enhance the risk of developing cardiovascular diseases in obesity and type 2 diabetic patients (26). In type 1 diabetes patients, excess levels of glutamate in the brain can be used as an early marker of neurodegenerative diseases associated with diabetes (27). Based on the above evidence, we conclude that an increase in the expression of glutamate receptors and its activity in the stomach tissue may be one of the markers of tissue damage (motor or secretory cells) and even, the reciprocal increase in the expression of GABA receptors might in part compensate glutamate receptor overactivity in diabetic tissue of the gastric antrum. Therefore, glutamate receptor antagonists with higher specificity might be a promising therapeutic strategy for the treatment of type 2 diabetes and metabolic syndrome and their complications. Magnesium is a NMDA antagonist and the diabetic rats treated with magnesium, in our study, significantly reduced gastric GABA receptor overexpression and NOS-1 reduction, suggesting that NMDA receptors may contribute partly to the positive impact of magnesium on gastric abnormality in the diabetic rats. In line with our study, Jan Marquard et al. (2015) found that NMDA receptor blockade by dextromethorphan increases glucose tolerance in mice, and proposed that NMDAR antagonists might be beneficial adjuncts in diabetes management (28). Additionally, memantine, an NMDA receptor inhibitor, reduced the susceptibility to hepatic steatosis in obese mice (29). Mechanistically, NMDA receptor activation impairs fatty acid, and thus inhibiting glutamate receptors may be important in attenuating tissue damage caused by chronic diabetes. Previous studies indicated a decreased NOS receptor activity in diabetic patients. Moreover, one of the confirming indicators of gastric injuries in diabetes is the assessment of NOS-1 expression. Given that the function of activated nNOS depends on the NMDA receptor and that nNOS plays a role in diabetic gastropathy, it can be concluded that the NMDA receptor may also contribute to diabetic gastropathy (4). This is supported by the study indicating that the NMDA receptor can regulate gastric acid secretion (30). Taken together, we showed a substantial decline in gastric nNOS accompanied by altered expressions of GABA and NMDA receptors in diabetic rats. Treatment with magnesium and insulin restored the level of NOS-1 and GABA receptors to the control values in the stomach of diabetic rats. Our findings propose that magnesium and insulin might play a significant role in improving abnormalities caused by diabetes, possibly through modulating the expression of NOS-1 level and GABA receptors. Considering the beneficial role of herbal medicine in attenuating different diabetic mellitus complications (31–33) and that herbal supplements containing magnesium have been associated with improvement of metabolic control and insulin resistance in non-diabetic overweight patients (15), we suggest a combined treatment with herbal and magnesium supplements in relieving diabetes complications, including gastropathy in future studies.

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Authors' Contribution
Study concept and design: M. G and N. M.
Administrative, technical, and material support: H. R. and H. S.
Acquisition of data: H. S.
Analysis and interpretation of data: M. G. and N. M.
Drafting of the manuscript: H. S.
Critical revision of the manuscript for important intellectual content: N. M. and M.G.

Ethics
All protocols were permitted by the Committee of Experimental Animal Administration of Isfahan University of Medical Science (IR.MUI.MED.REC.1400.737) based on National Institutes of Health guidelines.

Conflict of Interest
Not Applicable

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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