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EXPERIMENTAL STUDY

The relationship between ghrelin and inflammation in diabetic rat stomach

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ABSTRACT
BACKGROUND: Ghrelin is a hormone that regulates the digestive system, as well as has immunomodulating effects. The aim of this study is to explain effects of ghrelin on inflammation and oxidative stress parameters in the stomach.
METHODS: Male Sprague Dawley rats 8–10 weeks old (n = 21) were randomly divided into three groups as control, type 2 diabetes (T2DM) and diabetes and given exogenous ghrelin (T2DM+Gh). The daily feed and water intake of the animals were measured. The levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), and interleukin-10 (IL-10) mRNA in tissues were analyzed using RT-PCR technique. Ghrelin and nuclear factor-κβ (NF-κβ) peptides were detected by immunohistochemistry.
RESULTS: T2DM group had a significant increase in water intake when compared to control group. T2DM group had significantly higher levels of IL-6, IL-1, and IL-10 mRNA expression than control group. IL-1β and IL-10 mRNA expression were significantly lower in T2DM+Gh group than in T2DM group. In T2DM group, NF-κβ was higher than in control group, but it was lower in T2DM+Gh group. In terms of oxidative stress, there were non-significant changes.
CONCLUSION: According to our findings, exogenous ghrelin intake was found to be highly effective in reducing inflammation in stomach tissue with type 2 diabetes *(Tab. 1, Fig. 3, Ref. 33)*. Text in PDF *www.elis.sk*
KEY WORDS: ghrelin, rat, type 2 diabetes, stomach, inflammation.

Introduction

Diabetes is a multisystemic disease that affects not only the pancreatic tissue but also the entire metabolism, especially the digestive system. It is one of the major causes of morbidity and mortality globally. Diabetic patient population is expected to increase dramatically in the next decades. It is estimated that the number of 537 million adults living with diabetes will increase to 783 million by 2045. Type 1 (T1DM) and type 2 diabetes (T2DM) are the two most common types. T1DM diabetes is an autoimmune disease in which the insulin-producing organ, the pancreas, is attacked and destroyed by patients' T cells. T2DM is a chronic disease characterized by a combination of genetic, environmental, and lifestyle factors. It is often referred to as a lifestyle-related chronic disease (1). Hyperglycemia and insulin resistance characterize T2DM, a chronic metabolic disease. In addition, oxidative stress and endoplasmic reticulum stress are developmental mechanisms of T2DM. The inflammatory response can be triggered against all of these mechanisms. Inflammation is a common feature of diabetes' natural history and levels of inflammatory biomarkers the most of which are secreted by adipocytes are associated to both diabetes and cardiovascular disease (2). The adaptive and innate immune systems are both important in glycemic control regulation (3). Recent research shows that chronic inflammation is associated with insulin resistance and cell dysfunction, and that has a role in the development of T2DM. In addition, it is suggested that plasma levels of TNF-α, IL-1β and IL-6 are increased in T2DM (4). Discovered by Kojima et al ghrelin is mainly produced as an endogenous ligand for gastric GHS-R (growth hormone secretagogue receptor) (5). In vitro and in vivo, it controls growth hormone secretion and appetite regulation in a dose-dependent manner (6). Ghrelin is a 28-amino-acid peptide hormone that is primarily produced in the stomach. It has antioxidant properties and immunomodulatory effects and also regulates the digestive system. The relationship between ghrelin and the immune system has been demonstrated by the effects of ghrelin treatment on inflammatory responses (7). They also reported for the first time ghrelin mediated nuclear factor-κβ (NF-κβ) inhibition by anti-inflammatory effects on endothelial cells (8). NF- κβ has a crucial role in controlling the synthesis of several key proteins involved in the activation and maintenance of the inflammatory state (2). The levels of inflammatory factors are associated to NF- κβ, a nuclear transcription factor. Inactive NF-κβ is often located in the cytoplasm. When NF-κβ is

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phosphorylated, it causes the production of inflammatory cytokines including TNF- α, IL-β, and IL-6, which have a synergistic effect on the inflammatory response and tissue damage (9). Ghrelin hormone is expressed in human monocytes and T cells and has anti-inflammatory effects, according to studies. Ghrelin inhibits the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (10). Ghrelin may be a therapeutic agent in inflammatory diseases (11). It reduces the increase in circulating TNF-α, IL-1β and IL-6 induced by LPS (lipopolysaccharides) (12). IL-1β is a key regulator of ghrelin expression during inflammatory processes (13). It is reported that ghrelin regulates pro-inflammatory cytokine expression in human and murine T cells (14). Anti-inflammatory marker as IL-10 and total anti-oxidant status were associated with ghrelin expression (15). Anti-oxidation effects of ghrelin have been shown by regulation of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels (16).

Materials and methods

Experimental design

8–12 weeks old male Sprague–Dawley rats that were obtained from the Experimental Medical Research Institute, Istanbul University. The rats were fed ad-libitum and maintained in a cycle of 12 h of light, 12 h of darkness at ± 21 °C. All the experiments were conducted in accordance with the guidelines of the Local Ethics Committee for Research on Animals, Istanbul University (2018/55). The rats were grouped into three categories at random. Group I (n = 7), control, a single dose of saline was given to rats intraperitoneally. Group II (n = 7), T2DM, the animals received 10 % fructose in their water for two weeks, then a single dose of streptozotocin (STZ) (40 mg/kg, Sigma-Aldrich, St. Louis, MO, USA, S0130) intraperitoneally. 72 hours after STZ injection, blood samples were obtained from the tail of rats for glucose measurement. Rats whose fasting blood glucose levels were more than 200 mg/dL, were accepted as diabetic. In Group III (n = 7), T2DM+Gh the diabetic rats were treated with ghrelin (25 mg/kg/day) for two weeks.

At the end of the experiment, the rats were anaesthetized under ketamine hydrochloride (50 mg/kg, Ketalar, Pfizer) and xylazine hydrochloride (10 mg/kg, Rompun, Bayer). Blood and stomach tissues were collected. Fundus tissues were taken and put to 10 % neutral formalin to be fixed. The other separated tissue was fixed with liquid nitrogen and put at –80 ° C until the experiment.

Real-Time PCR (Polymerase chain reaction)

Protocol of Hibrigen kit was implemented for RNA isolation from stomach tissues (Cat No: MG-RNA-01). Master mix and RNA were added to the cDNA reaction for each sample. Incubated 10 ,at +25 °C, 120‘ at +37 °C, then 5 ,at +85 °C for the experiment of PCR. The changes of IL-6, IL-1β, TNF-α and IL-10 mRNA expression levels in the experimental groups were analyzed by real time PCR method. As a positive control, beta actin was used.

Immunohistochemistry

Sections were approximately 4 μm thick. All sections were deparaffinized using toluene and then rehydrated in decreasing concentrations of alcohol. Ghrelin antibody with 1:30 dilution (H-031-30, phoenix Pharm. Inc, USA) and NF-κβ antibody with 1:50 dilution (Santa Cruz sc-8414) were used for staining according to the streptavidin-biotin-peroxidase method. Histostain plus IHC kit (Invitrogen 85-9643) and AEC substrate kit (Invitrogen 00-2007) were implemented according to the manufacturer protocol.

Biochemical analysis

The stomach tissue samples were homogenized in cold 0.9 % NaCl and made up to 10 % homogenate. The homogenates were centrifuged and clear supernatant fractions were used for the biochemical analysis. The protein content was determined by Beutler's method using bovine serum albumin as standard (17). Glutathione (GSH), lipid peroxidation (LPO) and protein carbonyl (PCO) levels in stomach homogenates were estimated according to Beutler's, Ledwozyw's and Reznick and Packer's methods, respectively (17–19).

Statistical analysis

Statistical analysis was conducted using SPSS 21.0 software program. Statistics of the data were expressed as the mean ± standard error of the mean (SEM). Immunopositive cells in tissue sections were counted randomly per 10 areas. The section was observed with a light microscope (Nikon Eclipse 80i, Melville, NY, USA).

Data were evaluated for statistical significance using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant. Graphs were drawn using Graphpad Prism 8.0 program. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Results

Blood glucose, feed and water measurement

Mean fasting blood glucose values of all three groups were 82.14 ± 4.72 for control group, 340.33 ± 19.49 for T2DM group and 328.2 ± 53.19 for T2DM+Gh group (20).

Comparing at the end of the experiment the daily feed of rats between groups, there was no significant difference between the

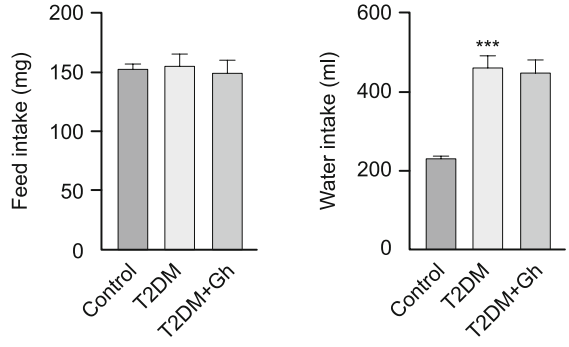

Fig. 1. Average daily feed amount of rats. Significant increase in water intake in the T2DM group compared to the control group (* p < 0.001). The results were evaluated as mean ± SEM.**

amount of feed. The daily water intake of the animals was followed and the difference among all groups was found to be statistically highly significant (PANOVA = 0.000). When control group and T2DM group were compared, a statistically significant increase was observed in T2DM group compared to control group (Fig. 1).

IL-6, IL-1β, TNF-α and IL-10 mRNA expression levels

When IL-6 mRNA levels, one of the pro-inflammatory cytokines, were compared among all groups, the fold changes were found to be statistically significant (PANOVA = 0.0286). T2DM compared with control group showed a statistically significant increase (p = 0.026). The decreased mRNA level was not statistically significant (p = 0.478) when T2DM group was compared with T2DM+Gh treatment group. According to IL-1β mRNA results, the fold changes between all groups were found to be statistically highly significant (PANOVA < 0.000). IL-1β mRNA levels were increased very significantly in T2DM group compared to control group (p = 0.000). Similarly, a highly significant decrease was found in T2DM+Gh group compared with T2DM group (p = 0.000). TNF-α mRNA fold changes were found not to be significantly different among all groups (p > 0.005). But there was a weak decrease of fold changes in the treatment group compared to T2DM group. In addition, control and treatment groups showed similar fold changes in TNF-α. The changes of IL-10 mRNA levels were statistically highly significant among all groups (PANOVA = 0,000). When the groups of control and T2DM were compared, a statistically significant increase was found in T2DM group mRNA level (p = 0.000). Additionally, there was a statistically highly significant decrease in treatment group compared with T2DM group (p = 0.000) (Fig. 2).

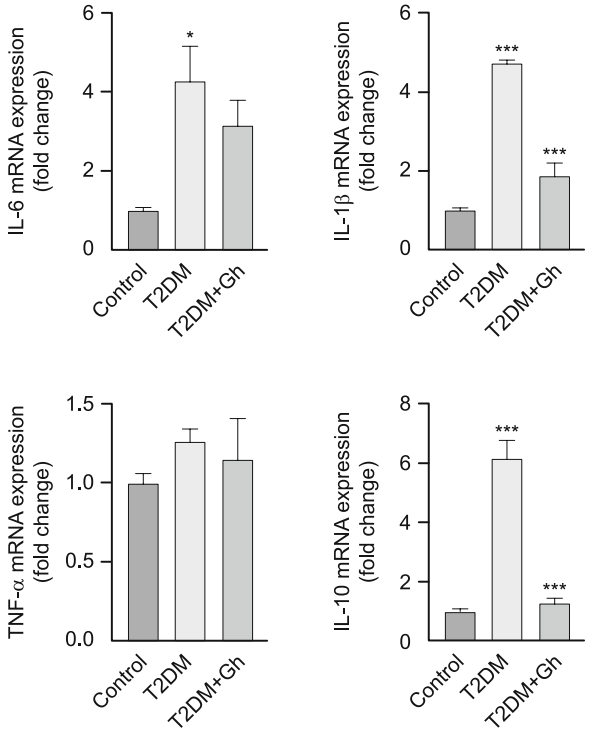

Fig. 2. An increase in interleukin-6 (IL-6) mRNA expression level in the type 2 diabetes (T2DM) group compared to the control group (* p < 0.05), a significant increase in interleukin-1β (IL-1β) mRNA level in the T2DM group compared to the control group and significant decrease in the type 2 diabetes + ghrelin (T2DM+Gh) group compared to the T2DM group (* p < 0.001), a significant increase in interleukin-10 (IL-10) in the T2DM group compared to the control (*** p < 0.001) and a significant decrease in the T2DM+Gh group (*** p < 0.001). The results were evaluated as mean ± SEM. ***p < 0.001**

Tab. 1. The changes in biochemical parameters.

Mean±SEM	Control	T2DM	T2DM+Gh
MDA	0.10±0.05	2.75±0.77	0.02±0.00
GSH	0.58±0.16	0.49±0.08	0.43±0.06
PCO	59.45±8.80	72.70±16.96	43.04±3.65

Immunohistochemical analysis of the ghrelin and NF-κβ

Changes between rat fundus ghrelin positive cell numbers were compared by ANOVA test. Among all groups no statistically significant differences were found (PANOVA = 0.1574). The number of NF-κβ positive cells in the fundus sections was statistically significant when comparing all groups (PANOVA = 0.000). It was found that the number of NF-κβ positive cells increased very significantly in T2DM group when compared to control group (p = 0.000). When T2DM and T2DM+Gh groups were compared, T2DM+Gh group showed a significant decrease (p = 0.0057). Nuclear signals of NF-κβ were higher in T2DM group compared to control and T2DM+Gh groups. Whereas, nuclear and cytoplasmic NF-κβ signal localization were seen less in T2DM+Gh group compared to T2DM group. All numbers of immunopositive cells changes are shown in Figure 3.

Biochemical analyses

When MDA, GSH and PCO determinations were evaluated between the groups, no statistical significance was found according to the ANOVA test (PANOVA = 0.157, PANOVA = 0. 4391, PANOVA = 0. 277). There was a decrease of MDA levels in treatment group, compared to T2DM group. Similarly, the same insignificant decrease was observed for GSH and PCO levels in the treatment group compared to T2DM group (Tab. 1).

Discussion

The anti-inflammatory effects of ghrelin, generally known as the hunger hormone, in T2DM disease was investigated in this study. In the fasted state, doubling of the plasma ghrelin level and decrease after the meal showed that ghrelin directly affects nutrition (21). In an initial study on animals, it was stated that although ghrelin does not have adipogenic effect on water intake, it is not limited to food intake but may also include changes in water intake (22). Considering that ghrelin is a hormone that provides a protective mechanism against hunger, it can be said that there is no effect in terms of feed intake between T2DM and T2DM+Gh groups in the study. The increase in water intake in T2DM and

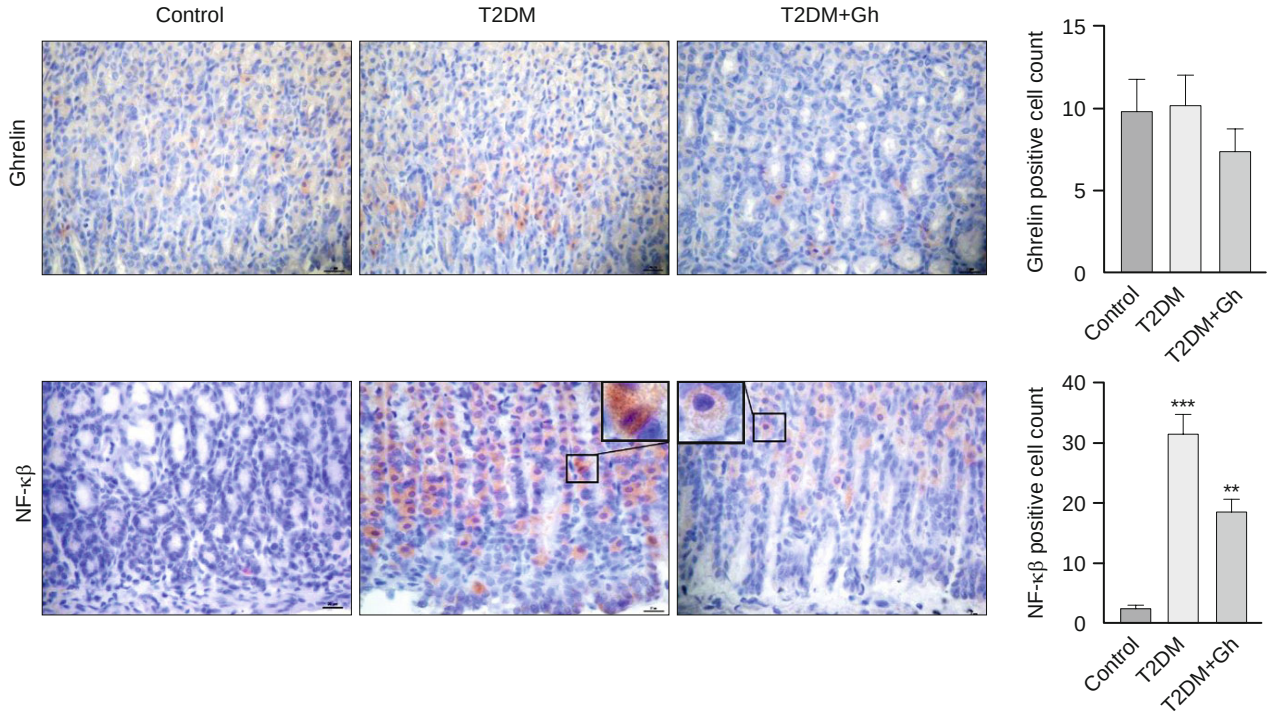

Fig. 3. Ghrelin and nuclear factor-κβ (NF-κβ) positive cells were demonstrated at x40 magnification. NF-κβ significantly increased in the type 2 diabetes (T2DM) group compared to the control group (* p < 0.001), and significantly decreased in the type 2 diabetes + ghrelin (T2DM+Gh) group compared to T2DM (** p < 0.01). Also averages were given as mean ± SEM. NF-κβ nuclear or cytoplasmic signals are shown as x100 magnification. Streptavidin-biotin-peroxidase technique, counterstain Hematoxylin.**

T2DM+Gh groups compared to control group may be associated with the effect of diabetes rather than ghrelin.

T2DM is a multifactorial disease and is one of the leading causes of early death (23). Researchers have emphasized the importance of elevated TNF-α and IL-6 level which is indicative of inflammation in the development of T2DM. Phosad et al (24) studied the pro-inflammatory cytokines of the IL-6 increase to and their association with the development of T2DM. T2DM individuals serum IL-6 and TNF-α expression were reported to be higher as compared with healthy individuals (23). In another study, it was reported that TNF-α and IL-6 inflammatory cytokines were higher in individuals with diabetes compared to healthy individuals, while the level of the anti-inflammatory cytokine IL-10 was low. In addition, with sitagliptin treatment, while the level of IL-10 increased significantly, the level of inflammatory cytokines decreased (25). Madison et al (11) suggested that the IL-1β receptor may be a therapeutic agent in inflammatory diseases. In another study comparing healthy and diabetic individuals, it was suggested that IL-1β and TNF-α levels significantly increased in the group with type 2 diabetes (26). The presence of ghrelin receptors on immune system cells supports their effect on inflammation (14). Studies on the anti-inflammatory effects of ghrelin have demonstrated that it decreases the expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α. (8,10,27). In the present study, IL-6, IL-1β, and TNF-mRNA expression levels in stomach tissues belonging to T2DM group were found to be higher than in control group. It was also observed that administration of exogenous ghrelin to T2DM group caused a decrease in inflammatory cytokines, especially IL-1β and IL-6 mRNA expression levels. Lontchi-Yimagou et al (2) emphasized that targeting inflammatory pathways may be a part of strategies to prevent diabetes and its related complications. The anti-inflammatory role of ghrelin may be due to its direct effect on T lymphocytes and monocytes, which inhibits the expression of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. It has been reported that ghrelin intake is effective on pain and obesity by increasing the expression of IL-10, one of the anti-inflammatory cytokines (28). IL-10 mRNA level increased in parallel with inflammatory cytokines in T2DM group. However, the reduction of IL-10 mRNA level with the application of ghrelin explains the immune regulatory role of ghrelin. Immune system cells change immune responses and cytokine expression in response to changes in metabolism and energy status (24). In our study, we can explain the increase of both anti-inflammatory and pro-inflammatory cytokines in gastric tissue by the effect of fructose in T2DM model created with fructose. Inflammation-related reductions in ghrelin are associated with IL-1β which is a key mediator of the inflammatory response (13). The marked reduction of IL-1β increase with ghrelin indicates that ghrelin treatment has a therapeutic effect on T2DM rat gastric tissue.

The activation of the NF-signaling pathway occurs as a result of beta cell dysfunction in the development of T2DM (29). Li et al (8) reported for the first time that ghrelin may have an anti-inflammatory effect by inhibiting NF-κβ activation in human endotelial cells. According to the results of studies conducted on transgenic animals, suppression of NF-κβ activity caused impaired glucose uptake, oxidative damage and expression changes of genes involved in insulin exocytosis (30). In the acute lung injury model they created in Sprague-Dawley rats, Zhang et al (31) reported that exogenous ghrelin significantly reduced the inflammatory response and the damage caused by inflammation. Ghrelin regulates the pro-inflammatory response as determined by plasma IL-6 and TNF-levels, and has effects on inflammation by inhibiting the activation of the Iκκ / Iκβα / NF-κβ signaling pathway (31). In our study, according to the significant decrease in the number of NF-κβ positive cells in type 2 diabetic rat gastric tissue with the administration of ghrelin supports that ghrelin reduces inflammation through the NF-κβ pathway. NF-κβ binds to the kappa response element and causes inflammatory mediators to be produced. The localization of NF-κβ is used as a molecular sign of the inflammatory response. T2DM group had higher NF-κβ nuclear signals. Nuclear and cytoplasmic NF- κβ were lower in T2DM+Gh group as compared to T2DM group. Therefore, it may cause an increase in pro-inflammatory cytokines, one of the target genes of NF-κβ. The increased IL-6 and IL-1β gene expression levels in T2DM group are consistent with the increased nuclear signals of NF-κβ, according to our RT-PCR results. These findings are consistent with the results of IL-1β gene expression, one of the pro-inflammatory cytokines, in T2DM+Gh group. Lithium inhibits NF-κβ translocation from the cytoplasm to the nucleus and regulates inflammation, according to Makola et al (32). In the study, the increase in the number of nuclear signals compared to cytoplasmic signals in T2DM group may have increased the secretion of proinflammatory cytokines, which are NF-κβ target genes. Oxidative stress contributes to the development of T2DM through a variety of molecular mechanisms. Abdulmalek et al (33) reported that selenium nanoparticles had reduced cytokine expression and hepatic MDA levels in diabetic rats. Ghrelin also has beneficial effects on oxidative stress and inflammation as an anti-inflammatory hormone in various pathological conditions (14, 16).

MDA and PCO levels were decreased in T2DM+Gh group, but the difference was not significant. Finally, our findings suggest that ghrelin may regulate inflammatory processes through inflammatory markers such as IL-1β, IL-10 and NF-kβ, rather than oxidative stres.

Conclusion

In this study, the effect of T2DM on stomach in pro/anti-inflammation and oxidative stress states with ghrelin treatment was tried to be clarified for the first time. The decrease in pro-inflammatory cytokine expression levels after exogenous administration of ghrelin to diabetic rats supports that inflammation inhibition is associated with NF-κβ expression. The decrease in the expression level of anti-inflammatory and immunomodulatory IL-10 with ghrelin intake to a level close to control is a finding that supports the anti-inflammatory effect of ghrelin. In addition, the decrease in IL-1β expression level after ghrelin intake also supports the regulatory role of ghrelin during inflammation. The fact that there was no significant change in the numbers of the fundus ghrelin cells with exogenous ghrelin intake indicates that ghrelin has no effect on positive cells increase. The present study shows promise for new treatment pathways targeting the inflammation that contributes to the progression of T2DM. Among the anti-inflammatory agents that target inflammation, ghrelin may have significant potential in the future treatment of T2DM patients.

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