Effects of glucagon-like peptide-2 on bacterial translocation in rat models of colitis

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Background/aims: The aim of this trial was to study the role of glucagon-like peptide-2 in reducing bacterial translocation by virtue of its anti-inflammatory effects and ability to decrease intestinal permeability in rat models of inflammatory bowel diseases. On the basis of our results and those of other recent studies, we suggest a new treatment modality for colitis. To our knowledge, this is the first study of the effectiveness of glucagon-like peptide-2 on bacterial translocation, in treating an experimental colitis model.

Methods: Rats were randomized into 3 groups of 7 rats each-the control group, colitis group, and treatment group. On the 7th day after induction of colitis, the levels of tissue myeloperoxidase, serum tumor necrosis factor-alpha, and plasma endotoxin were measured. Tissue samples were obtained from the liver, spleen, and mesenteric lymph nodes for evaluating bacterial translocation.

Results: Bacterial translocation in samples of the liver, spleen, mesenteric lymph nodes, and portal and systemic blood obtained from the treatment group was lower than that in samples obtained from the colitis group (p<0.05). The levels of tissue myeloperoxidase, serum tumor necrosis factor-alpha, and plasma endotoxin in the treatment group were significantly lower than those in the colitis group (p<0.05).

Conclusions: In experimental colitis models, which were induced using trinitrobenzene sulfonic acid in ethanol, glucagon-like peptide-2 treatment reduced inflammation and bacterial translocation from the intestinal mucosa. Our results indicate that glucagon-like peptide-2 is a potential agent for treating colitis; however, extensive trials are needed to confirm our results.

Key words: Glucagon-like peptide-2, experimental colitis, bacterial translocation

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Amaç: Bu çalışmadada glukagon benzeri peptid 2' nin inflammatur barsak hastalıklarında gösterdiği antiinflamattuar etki ve intes- tinal permeabiliyeti azalma yönündeki etkisini, bakteriyel translokasyon üzerinde doğrudan ve gidecek sonuçu olmayan etkili lehinde artırılmış ve bu çalışmanın doğruluğunu sonuçlar üzerinde, kolit tedavisinde kullanılan en potansiyel tedavi modülasyonun literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürdeki literatürdeki literatırdeki literatürü
INTRODUCTION
Inflammatory bowel diseases (IBDs) are a group of diseases that affect the gastrointestinal system (GIS) and deteriorate the quality of life. Ulcerative colitis (UC) and Crohn’s disease (CD) are two similar diseases that are generally studied as IBDs. Although the cause of IBDs has not yet been understood, they are thought to be multifactorial diseases. The most accepted theory is that in genetically susceptible individuals, uncontrolled, diffuse inflammation in the GIS causes changes in the intestinal barrier functions under the influence of environmental factors, thereby leading to IBDs. The incidence of IBDs has progressively increased in the last decade. Oxidative stress is thought to trigger IBDs. However, the harmful effect of reactive oxygen molecules has not been completely understood. Human and animal trials have shown that increased oxidative stress in the intestinal mucosa; activation of intracellular antioxidant enzymes such as glutathione peroxidase (GPx), myeloperoxidase (MPO), and superoxide dismutase (SOD) in the colonic mucosa; and reduced antioxidant levels are associated with IBDs (1,2). The upregulation of gut mucosal cytokines such as tumor necrosis factor-alpha (TNF-α) and oxidative stress have been related to IBDs such as UC and CD (3). Oxidative stress and oxidative cellular damage also play an important role in the pathogenesis of chronic UC and the associated carcinogenetic process (4).

The structure of the gastrointestinal epithelium is such that it can regulate the water balance and present a barrier between the intestinal lumen and interstitial space. In IBDs, the intestinal mucosal barrier is destroyed because of inflammation and ulceration. As a result, both enteric bacteria and intraluminal substances can enter the extra-intestinal regions. This bacterial translocation can cause secondary bacterial infections such as abscess or peritonitis. Endotoxemia and other related diseases and increase in epithelial permeability are probably the early signs of disease relapse (5).

In this study, we aimed to determine the anti-inflammatory effects of glucagon-like peptide-2 (GLP-2) and its effect on bacterial translocation and to evaluate whether GLP-2 can be used for treating colitis by performing pathophysiological examinations of colitis models.

MATERIALS AND METHODS

Animals
Male Wistar albino rats weighing 225–290 g were fed a balanced rodent diet and housed under stable environmental conditions. Rats were only fed 12 hours (h) before the experiments. All the experimental procedures performed on the rats used in the animal trials were in accordance with the guidelines of the National Health Institute and were approved by the Local Animal Care Committee.

Experimental Design
In this study, 21 Wistar albino rats (28-32 weeks old) were divided into the following three groups: Group 1 (control group), rectal saline; Group 2 (colitis group), colitis induced by trinitrobenzene sulfonic acid in 50% ethanol (TNBS-E) followed by no treatment; Group 3 (treatment group), colitis induced by TNBS-E followed by GLP-2 treatment.

Experimental Procedure
Rats were sedated with halothane and kept under spontaneous ventilation throughout the procedure. After sedation, the rats were weighed. For induction of colitis, 5F plastic cannulas were inserted rectally, and 50% TNBS-E (15 mg/0.3 ml) was injected into each rat via the cannula. To prevent regurgitation of the chemical, the rats were placed in the supine position.

The rats in the control group received intracolonic injections of saline. For 7 days after the induction, rats in the treatment group were subcutaneously injected with GLP-2 (50 μg/kg) daily for 7 days, whereas rats in the control and colitis groups were subcutaneously injected with saline. All rats were injected after topical application of 10% povidone-iodine.

The rats were fed with standard rat food during these 7 days. After 7 days, all rats were operated. During the operation, rats were anesthetized by intraperitoneal injections of ketamine-HCL (10 mg/kg; Ketalar, Eczacibasi, Turkey) and xylazine (3 mg/kg; Rompun 2%, Bayer, Germany). After shaving the operating field, it was sterilized with 10% povidone-iodine. A surgeon performed all procedures under sterile conditions. After a midline abdominal incision, 2-ml samples of both portal and systemic blood were drawn for biochemical analysis and culturing. Samples were obtained from the liver, spleen, and mesenteric lymph nodes (MLNs) for detecting bacterial translocation. Tissue samples from the colon were examined and were assayed for MPO. At the end of the operation, all rats were sacrificed by administration of a high dose of ether.
Quantification of Serum TNF-α Levels
For measurement of serum TNF-α levels, serum samples were obtained by centrifuging blood samples at 4000 rpm for 10 minutes (min). Samples were stored at -70°C in Eppendorf tubes. Serum TNF-α levels were measured using TNF-α ELISA kits (TNF-α Rat ELISA Kit, Invitrogen, USA) and are expressed in pg/ml.

Quantification of Plasma Endotoxin
The levels of endotoxin in plasma derived from portal venous blood were measured using a quantitative modification of the Limulus amebocyte lysate test, and are expressed in pg/ml.

Evaluation of Bacterial Translocation
Blood samples obtained during the operation were cultured in Pedi-BacT culture bottles, and bacterial growth was detected using an automated culture system. The bottles containing the culture media were incubated at 35-37°C and continuously shaken and followed at 10-min intervals. The concentration of CO₂ produced in the culture media was continuously measured by a colorimetric instrument, the output of which was detected by reflectometers. The system was programmed to provide visual and audio alerts when CO₂ was produced in the culture media. Next, samples from the blood cultures that were positive for CO₂ production were passaged on blood agar and eosin methylene blue (EMB) agar and incubated at 37°C for 24 h. Finally, the microorganisms were identified using conventional methods. Blood cultures in which CO₂ production had not detected by 7 days were considered negative cultures. Tissue samples were cultured in sterile bottles containing brain–heart media, and these bottles were incubated at 37°C for 24-48 h. Samples from positive cultures were passaged on blood agar and EMB agar and incubated at 37°C for 24 h. The microorganisms obtained from the positive cultures were identified using conventional methods.

Evaluation of Colonic Injury
During the operation, the distal colon was resected and dissected longitudinally. The fecal material was removed from the colon using saline. These colonic segments were examined by a pathologist who was unaware of this trial.

Scoring Based on Macroscopic Findings of the Colon
Macroscopic findings of the colonic mucosa were scored on the basis of the classification system developed by Bertevello et al. (6). Macroscopic morphological features according to five groups and their scores are given in Table 1.

Scoring Based on the Microscopic Findings of the Colon
Tissue samples of the colon were fixed in 10% formaldehyde and stored in 70% ethanol. The tissues were cut into 4-μm slices after they were embedded in paraffin blocks. After hematoxylin-eosin staining, the tissue slices were examined under a light microscope by a pathologist who was unaware of the randomization of rats. Samples were evaluated for colitis and tissue damage based on serial contacts with a lancet. The microscopic findings were scored on a scale of 0-3 (7) (Table 2).

Quantification of MPO Activity in Colonic Tissue
Myeloperoxidase (MPO) activity was measured in colon homogenates using the Rat MPO ELISA kit (Cell Sciences, USA) as described by Krawisz et al. (8). According to their method, the colonic mucosa was homogenized in a solution containing potassium phosphate buffer (pH 6.0) and hexadecyl trimethyl bromide. The homogenate was exposed 3 times to a freeze/thaw cycle. The homogenate was then centrifuged at 40,000 g for 15 min at 4°C. O-dianisidine-H₂O₂ buffer was added to the supernatant, and changes in absorbance were measured for 2 min at 460 nm. One unit of MPO activity is defined as the amount of MPO required to degra-

<table>
<thead>
<tr>
<th>Grade</th>
<th>Finding</th>
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<tbody>
<tr>
<td>0</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Erythematous areas without edema, congestion, and superficial ulceration</td>
</tr>
<tr>
<td>2</td>
<td>Superficial linear ulceration (7-15 mm); depressed, erythematous or dense areas, and granular zone</td>
</tr>
<tr>
<td>3</td>
<td>Irregular ulceration (15-15 mm); presence of normal islands around the ulcerated mucosa and presence of edematous mucosa</td>
</tr>
<tr>
<td>4</td>
<td>Diffuse irregular and multiple ulcerations (&gt;45 mm); thin intestinal wall with granular and irregular base</td>
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</table>
Statistical Analysis

Data was analyzed using the Statistical Package for the Social Sciences (SPSS) 15.0 and SigmaStat 3.5 statistical software packages. Variability was evaluated using the Kolmogorov-Smirnov test. For intergroup comparisons, one-way analysis of variance (ANOVA) was performed for variables with normal distribution.

For the Tukey’s test for multiple comparisons was performed for the groups for which the results of the one-way ANOVA were significant.

Kruskal-Wallis analysis was performed for the variables that did not follow a normal distribution. The Student-Newman-Keuls test for multiple comparisons was performed for the groups for which the results of the Kruskal-Wallis analysis were significant. Two-rate tests were performed for assessing bacterial translocation. Values are expressed as average±standard error. P values <0.05 were considered as statistically significant.

RESULTS

Colonic Injury

Macroscopic findings

Findings of the macroscopic and microscopic examination of the colonic tissues are listed in Table 3. In the GLP-2-treated group, we observed mild injury on the colonic epithelial surface and moderate injury on the mucosa, but we did not observe any transmural injury. The most severe macroscopic injuries were observed in the colitis group (mean: 1.42), less severe injuries in the treatment group, and no injuries in the control group (p<0.05).

Microscopic findings

Microscopic histopathological examination showed that the rats in the control group had normal tissues. In rats treated with GLP-2, mild injury and inflammation were noted, whereas in rats with colitis, injuries with transmural infiltration of neutrophils, monocytes, and lymphocytes were observed (p<0.05; Table 3).

Quantification of Serum TNF-α

On the 7th day, the levels of TNF-α in the colitis and treatment groups were higher than those in the control group (p<0.05, Figure 1). The level of serum TNF-α in the treatment group was lower than that in the colitis group (p<0.05; Figure 1).

Quantification of plasma endotoxin

On the 7th day, the levels of plasma endotoxin in the colitis and treatment groups were higher than those in the control group (p<0.05, Figure 2). The level of plasma endotoxin in the treatment group was less than that in the colitis group (p<0.05; Figure 2).

Quantification of MPO

The MPO levels in the control and treatment groups were lower than those in the colitis group (p<0.05, Figure 3).

Quantification of bacterial translocation

No bacterial growth was detected in samples obtained from the control group. In the colitis group,
increased translocation of bacteria to the liver, spleen, MLNs, and portal system was observed. Compared to the colitis group, the treatment group showed less bacterial translocation (p<0.05). The numbers of rats showing bacterial translocation to different regions are listed in Table 4. Species that were identified in the blood cultures included *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Enterobacter agglomerae*.

**DISCUSSION**

Inflammatory bowel diseases (IBDs) include UC and CD, which are of unknown cause, characterized by acute or chronic inflammation. IBDs increase the risk of colorectal cancer (9). The incidence of UC peaks for two different age groups, 15-30 years and 50-70 years, and only 18% of the UC patients are younger than 18 years (10). The incidence of IBDs differs geographically; it is low in Asia and South America (0.5/100,000 and 0.08/100,000, respectively). In the United States, the incidence of UC is 11/100,000, whereas that of CD is 7/100,000 (11). Although IBDs are rare, their impact on the quality of life, and their role in increasing the risk of colorectal cancer and other morbidities warrants further research for the development of new treatment modalities against IBDs. In addition, the fact that this disease is rare makes it difficult to study this disease and to investigate its treatment options. Failure to generate an acute inflammatory exacerbation in animal models is the most important factor hindering in vitro experiments on IBDs. On the basis of past experiences, we used TNBS, a known inducer of colitis, to induce colitis in rats. In a TNBS colitis model, chronic inflammation and ulceration develop because of hapten-triggered delayed-type hypersensitivity. In this model, colonic thickening and ulceration develop in the first 4 days and are observed until 8 weeks. This long duration allows the observation of inflammatory cells such as polymorphonuclear leukocytes, macrophages, lymphocytes, mast cells, and fibroblasts and increased connective tissue in the mucosa and submucosa. This property makes TNBS an ideal agent for inducing colitis (12-14).

The immune system, genetic factors, and environmental factors are thought to play a central role in the pathogenesis of IBDs (15,16). In particular, the association between IBDs and an increase in reactive oxygen species due to defects in the anti-
An oxidant system or phagocytic cell infiltration has been established (9). Therefore, the main pathophysiological mechanism for IBDs is the derangement in Th1 and Th2 activation or the imbalance between pro-inflammatory and anti-inflammatory cytokines. Th1 cells produce cytokines (TNF-α, interleukin-1 [IL-1], IL-2, IL-6, IL-8, IL-12, and interferon-γ [INF-γ]), which maintain the inflammatory response, whereas Th2 cells produce cytokines (IL-4, IL-10, IL-11, and IL-13) that depress the inflammatory response. Pro-inflammatory cytokines exacerbate inflammation, whereas anti-inflammatory cytokines have the opposite effect. A number of publications suggest therapies to suppress or decrease the effect of pro-inflammatory cytokines (17).

Glucagon-like peptide-2 (GLP-2) has different effects on the inflammatory process. Primarily, it inhibits gastric motility and increases intestinal absorption. It has an intestine-specific trophic effect and stimulates crypt cell proliferation, thereby indirectly suppressing apoptosis of mucosal epithelial cells. It enhances bowel growth and reduces the severity of colonic injury. It also reduces intestinal inflammation and has trophic effects on isolated neurons, so it can be a potent intestinotrophic growth factor with therapeutic potential for the prevention and treatment of an expanding number of gastrointestinal diseases (18-20). In addition, GLP-2 has different effects on the inflammatory cascade; it decreases intestinal motility, bacterial translocation, and pro-inflammatory cytokine expression in acute and chronic inflammation. This decrease is mediated via G protein-bound receptors. This indicates that GLP-2 exerts its effects via neuronal and endocrine cells instead of enterocytes and that systemic effects of GLP-2 are indirectly mediated by secondary signals (21).

Table 4. Bacterial translocation in the 3 groups

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control group</th>
<th>Colitis group</th>
<th>Colitis + GLP-2 Group</th>
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<tbody>
<tr>
<td>MLN</td>
<td>0</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Portal blood</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Systemic blood</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Bacterial translocation for the 3 groups was compared using the ratio test. Tests to detect bacteria were performed for all rats (7 rats in each group) (p<0.05).

The properties of GLP-2 such as protection of the mucosa and its indirect anti-inflammatory effects have been studied extensively. However, the therapeutic effects of GLP-2 in colitis have not yet been studied. This study was designed to evaluate the possible role of GLP-2 in colitis treatment. To our knowledge, no trials to evaluate the role of GLP-2 in treating colitis have been reported in the literature. The results of our pathophysiological examinations show that GLP-2 decreases bacterial translocation by virtue of its anti-inflammatory effects and ability to decrease intestinal permeability and that it may be used as a therapeutic agent for colitis. However, further experiments and clinical trials should be performed to verify these novel findings.

Another important aim of this study was to identify molecules that are involved in the inflammatory and anti-inflammatory processes. TNF-α, an important determinant of inflammation in IBDs, is accepted as the key molecule for monitoring the inflammatory process. Studies have suggested that oral administration of TNF-α antibodies can be used to efficiently treat experimental colitis models. However, all previous studies on rat colitis models observed an increase in TNF-α levels. For example, Kucuk et al. (23) observed the effect of Met-RANTES (methionylated-regulated on activation, normal T expressed and secreted) on bacterial translocation in a colitis models and found a progressive, parallel increase in TNF-α levels with inflammation and a decrease in TNF-α levels and bacterial translocation after Met-RANTES treatment. Akcan et al. (5) showed that melatonin decreased bacterial translocation and apoptosis in experimental colitis models induced with TNBS. In their experiment, the increased TNF-α levels and bacterial translocation decreased in animals with colitis after melatonin treatment. In addition, an increase in TNF-α levels with inflammation was observed in that study. We measured TNF-α le-
vels at day 7 after the induction of colitis in order to observe the changes in the inflammatory process. We observed that the TNF-α levels in the treatment group were lower than those in the colitis group (p<0.05). Additionally, bacterial translocation in the tissue and blood samples of the GLP-2 treatment group was lower than that in samples of the colitis group. Therefore, we conclude that GLP-2 reduces bacterial translocation through its intestinotrophic effect and by decreasing intestinal permeability and inflammation and that TNF-α shows a parallel route with all these processes.

Myeloperoxidase (MPO) activity has been used as a marker for neutrophil activity, particularly in inflammatory processes in the GIS. You et al. (24) showed that in rat colitis models, application of N-acetylcysteine decreased MPO levels in the intestinal tissue; this finding is consistent with that of our histological examination. Another study showed that the anti-inflammatory effect of GLP-2 is not correlated with IL-10 levels. In that study, GLP-2 treatment reduced weight loss, inflammation, and MPO levels (25).

Similar to the previously reported results, our results showed that MPO levels in the colitis group were higher than those in the control group (p<0.05), and that GLP-2 treatment reduced MPO levels (p<0.05; Figure 3). Our microscopic and macroscopic histopathological examination showed a reduction in the inflammatory process in the GLP-2-treated rats. These findings suggest that GLP-2 affects the anti-inflammatory process in two ways—it reduces crypt cell proliferation and cellular apoptosis.

Another important aspect of IBDs is their infectious process. The destruction of the intestinal mucosal barrier due to inflammation and ulceration causes bacterial translocation, and the enteric bacteria enter the portal and extraintestinal systems via the intestinal mucosa (18). Failure of the mucosal immune system leads to an inflammatory process and delayed-type hypersensitivity (26).

Berg and Garlington (27) have described bacterial translocation as the movement of bacteria from the epithelial mucosa to the lamina propria and further to the MLNs and extraintestinal system. Bacterial overgrowth and translocation are due to physical damage to the intestinal epithelium, derangement of the normal intestinal flora, or defects in the immune system (23,26).

We measured plasma endotoxin levels and cultured tissue and blood samples to assess bacterial translocation in colitis models. TNBS-induced colitis dramatically increased bacterial translocation to the liver, spleen, MLNs, and portal system. In the treatment group, we observed a decrease in bacterial translocation.

We observed that plasma endotoxin levels in the colitis group were higher than in the control group and lower than that in the GLP-2 treatment group (p<0.05, Figure 2). This indicates that GLP-2 reduces bacterial translocation by decreasing lymphatic permeability.

Our results show that GLP-2 reduces bacterial translocation by virtue of its anti-inflammatory properties and ability to decrease lymphatic permeability. Further studies are needed to evaluate the use of GLP-2 as a new treatment modality for colitis. Further, the role of GLP-2 in suppressing the progression of colitis and its effects in both acute and chronic phases of colitis should be investigated in experimental and clinical trials.

REFERENCES