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The protective effect of infliximab on cisplatin-induced intestinal tissue toxicity

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Abstract. – OBJECTIVE: Cisplatin (CP) is a popular chemotherapeutic agent. However, high doses of CP may lead to severe side effects to the gastrointestinal system. The aim of this study was to investigate the protective effects of infliximab on small intestine injury induced by high doses of CP.

MATERIALS AND METHODS: The A total of 30 rats were equally divided into three groups, including sham (C), cisplatin (CP), and cisplatin + infliximab (CPI). The CP group was treated with 7 mg/kg intraperitoneal cisplatin, and a laparotomy was performed 5 days later. The CPI group received 7 mg/kg infliximab intraperitoneally, were administered 7 mg/kg cisplatin 4 days later, and a laparotomy was performed 5 days after receiving cisplatin. Histopathological and immunohistochemical analysis of small intestine tissue sections were performed, and superoxide dismutase, malondialdehyde, and TNF- levels were measured.

RESULTS: Histopathological evaluation revealed that the CP group had damage in the epithelium and connective tissue, but this damage was significantly improved in the CPI group ($p < 0.05$). In addition, these histopathological findings were confirmed by biochemical analyses.

CONCLUSIONS: These results suggest that infliximab is protective against the adverse effects of CP.

Key Words:
Cisplatin, Intestinal toxicity, Infliximab, Protective effects.

Introduction

Cisplatin (CP, cis-Diamminedichloroplatin II) is one of the major chemotherapeutic agents used to treat various types of cancer[1]. However, high doses of CP are not often administered due to strong side effects that affect the kidney and gastrointestinal tract[2,3]. Increases in reactive oxygen species (ROS) and free radical production have been shown to mediate the toxicity caused by CP[4]. Various agents have been used to inhibit the formation of free radicals, and in addition, they can be used to prevent or reduce the adverse effects caused by CP. However, it remains unclear as to whether the clinical use of these agents can cause histopathological tissue disorders[4-8].

Tumor necrosis factor alpha (TNF-α) is an apoptotic cytokine that is secreted by many cell types and can cause damage to cancer cells[9]. TNF-α induces cell death by causing cytochrome C to be released from the mitochondria, which leads to caspase-9 activation[10]. Caspase-9 activation increases paracellular permeability and induces cell swelling, leading to apoptosis. Cell culture studies have reported that drugs such as infliximab and adalimab inhibit these TNF-α induced effects[11].

CP induces apoptosis by increasing mitochondrial permeability through various mechanisms. These apoptotic effects may also play a role in the toxicity of CP and its ability to cause tissue damage[12]. Several cancer chemotherapeutic agents (such as CP) use apoptosis as their primary mechanism[13]. Caspases are mediators that play critical roles in apoptosis. It has been reported that the programmed cell death induced by CP is activated through Caspase-3[14]. Caspase-3 can be detected by immunohistochemical staining in the small intestinal mucosa and, therefore, any tissue damage caused by CP can be evaluated with this method.

Infliximab (Remicade®) is a TNF antagonist in the form of a monoclonal antibody that binds to TNF-α with high affinity and specificity[15,16]. Several *in vitro* studies have been conducted with human fibroblasts, endothelial cells, neutrophils, lymphocytes and epithelial cells and have shown that infliximab inhibits the functional activity of TNF-α[15,17]. Currently, infliximab is used to treat

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rheumatologic, gastrointestinal, dermatological, and ocular diseases. In patients with these diseases, infliximab has been shown to act by inhibiting inflammation and oxygen free radicals[15,18,19]. However, to our knowledge, there have not been any studies investigating the protective effect of infliximab on CP-induced intestinal injury.

In this study, we aimed to investigate the protective effect of infliximab on tissues and cells in the CP-induced intestinal injury model.

Materials and Methods

In this study, 7 month old adult male Wistar albino rats (200-250 g) were used. All animals were maintained and fed in the sterile experimental animals unit with a 12 hour light and 12 hour dark cycle, 55-60% humidity and an ambient temperature of 22 ± 3°C. Animals in all groups were provided unlimited standard laboratory food and tap water *ad libitum*. No special environment was created.

The study protocol was obtained from the Recep Tayyip Erdogan University Local Animal Ethics Committee (report number 2012.106.01.5 from 3-22-2012) and the study (number 2013/55) was approved by the Local Animal Ethics Committee of Karadeniz Technical University on 5-11-2013.

Experimental Groups

A total of 30 male Wistar albino rats were randomly divided into 3 equal groups: Sham (C), cisplatin (CP, Ebewe) and cisplatin + infliximab (CPI, Remicade). Rats from the sham group (C) underwent a laparotomy without any drug administration. Rats form the CP group were administered 7 mg/kg CP intraperitoneally 5 days before the laparotomy. Meanwhile, rats from the CPI group were injected intraperitoneally with 7 mg/kg of infliximab 4 days prior to receiving 7 mg/kg CP intraperitoneally. The rats in the CPI group underwent laparotomy five days after receiving CP.

Intestinal tissues were obtained from the rats in all three groups, labeled with a given code number and stored in special bottles containing 10% formaldehyde. After incubation in the fixative for approximately 24 hours, intestinal tissues were washed with running tap water for 4-6 hours, and were rinsed in a series of increasing concentrations of alcohol (50%, 70%, 80%, 96%, absolute alcohol) and xylene. Then, the tissues were passed through an automated tissue processor (Citadel 2000, Thermo Fisher Scientific Shandon, Loughborough, Leicestershire, UK) and were embedded into liquid paraffin. Tissues were cut 4-6 μm thick for routine hematoxylin-eosin staining and 3-4 mm thick for immunohistochemical staining. Selected areas were examined under a light microscope at different magnifications and images were taken.

Intestinal tissue sections were stained with hematoxylin-eosin and were examined under a light microscope. Pathological evaluations were performed as described by Chiu et al[20]: grade 0 (normal), grade 1 (moderate damage), grade 2 (severe damage) and grade 3 (very severe damage).

Sections cut for immunohistochemical staining were incubated twice in xylene for 20-30 minutes and were rinsed with series of alcohol (70-99%) followed by incubation in 3% H_2O_2 for 20 minutes. After washing with phosphate buffered saline (PBS), the sections were placed in citrate buffer solution and heated in an 800-Watt microwave in 5 minute intervals 4 times, and then incubated for 20 minutes in secondary blocking agent. Then, each preparation was incubated in anti-laminin antibody [(ab11575), Abcam plc, Cambridge CB4 0FL, UK] and anti-caspase 3 antibody [(ab4051), Abcam plc, Cambridge CB4 0FL, UK] at room temperature for 75 minutes with the appropriate dilution (1/200) of primary antibodies. The diaminobenzidine (DAB) solution was used as the chromogen and Mayer's hematoxylin staining was used for contrast coloring. PBS was used for the negative controls. The preparations were covered with the appropriate closing materials and then photographed.

Biochemical Evaluations

Blood was collected from the rat hearts and was centrifuged at 1500 rpm/10 minutes prior to biochemical analysis. The activity of Superoxide Dismutase (SOD) was measured using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the instructions provided by the manufacturer. Absorbance was measured at a wavelength of 450 nm using a plate reader. The SOD activity was reported as U/ml. The intra-assay and inter-assay coefficients of variation were 3.2% and 3.7%, respectively. The dynamic range for the SOD assay kit was 0.025-0.25 U/ml. Malondialdehyde (MDA) levels were measured by the double heating method

described by Draper and Hadley[21]. The principle of this method involves the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. The MDA levels are presented as mmol/L (Table I).

TNF-α was measured with an enzyme-linked immunosorbent assay (ELISA). We used a commercially available rat TNF-α ELISA kit (eBioscience, Vienna, Austria) and followed the instructions provided by the manufacturer. Absorbance was measured at a wavelength of 450 nm using an ELISA reader. The levels of TNF-α are presented as pg/ml. The intra-assay and inter-assay coefficients of variation were <5% and <10%, respectively. The limit of detection (LOD) for the TNF-α assay was 11 pg/ml. The obtained TNF-α values were divided by the protein concentration to obtain the final result, which was presented as pg/mg (Table II).

Statistical Analysis

Data were analyzed using the SPSS program (SPSS for Windows, v. 18.0, IBM, Chicago, IL, USA). The MDA, TNF-α, and SOD values were analyzed using a one-way ANOVA and were expressed as mean ± SD and mean ± standard error of the mean ($p < 0.05$ was considered significant). Multiple comparison tests (Post Hoc Tukey HSD parametric tests) were used to identify significant differences between groups. Assessment of histopathological status and intensity of immunohistochemical (IHC) reactions were expressed as medians (min-max). The Kruskal-Wallis test was used for inter-group analysis ($p < 0.05$ was considered significant) (Table III, IV).

Table I. Mean ± SD and statistical evaluations of SOD and MDA values.

Group	N	SOD Mean ± SD	MDA Mean ± SD
Control	10	33.45±3.40[a]	3.61±0.48[b]
Cisplatin	10	26.35±2.15[aa]	6.21±0.94[bb]
Cisplatin+ Infliximab	10	29.76±2.25[aaa]	4.89±0.81[bbb]

[a] Based on the Tukey HSD test there is a statistically significant difference in terms of SOD levels between the control group and the cisplatin group, ($p < 0.05$).
[aa] Based on the Tukey HSD test there is a statistically significant difference in terms of SOD levels between the control group and the cisplatin+infliximab group, ($p < 0.05$).
[aaa] Based on the Tukey HSD test there is a statistically significant difference in terms of SOD levels between the cisplatin group and the cisplatin+infliximab group, ($p < 0.05$).
[b] Based on the Tukey HSD test there is a statistically significant difference in terms of MDA levels between the control group and the cisplatin group, ($p < 0.05$).
[bb] Based on theTukey HSD test there is a statistically significant difference in terms of MDA levels between the control group and the cisplatin+infliximab group, ($p < 0.05$).
[bbb] Based on the Tukey HSD test there is a statistically significant difference in terms of MDA levels between the cisplatin group and the cisplatin+infliximab group, ($p < 0.05$).

Table II. The statistical analysis of TNF α levels.

Group	N	TNF-α Mean ± Std. Error of Mean
Control	10	239.10±20.30[a]
Cisplatin	10	316.24±10.68 [aa]
Cisplatin+ Infliximab	10	263.23±11.17 [aaa]

[a] Based on the Tukey HSD test there is a statistically significant difference between the control group and the cisplatin group, ($p < 0.05$).
[aa] Based on the Tukey HSD test there was no statistically significant difference between the control group and the cisplatin+infliximab group, ($p < 0.05$).
[aaa] Based on the Tukey HSD test there is a statistically significant difference between the cisplatin group and the cisplatin+infliximab group, ($p < 0.05$).

Results

Histopathology

The histopathological examination of sections obtained from group C (sham) with HE staining did not show any tissue or cellular damage, and structures with normal histological appearance and morphology were observed (Figure 1A). The crypts in the glandular epithelium were tightly arrayed cylindrical structures. The lamina propria characterized by reticular connective tissue contained lymphatics and capillaries and was surrounded by individual lymphocytes, plasma cells, and eosinophils, as well as granulocytes that were not grouped. High columnar absorptive cells stained with eosinophil were shown to have regular arrangement. In addition, villi were aligned in finger-like regular protrusions in the lumen, while the Goblet cells with oval nuclei were stained with basophilic staining (Figure 1A).

The histopathologic examination with hematoxylin- eosin staining showed that there was tissue and cellular damage in the CP group (Figure 1). The crypts had lost their cylindrical and tightly arrayed structures, and their lumens were enlarged. Vacuolization in the crypt epithelium, cell

Table III. Histopathological evaluation of the groups.

Groups	Capillary congestion	Epithelial sloughing	Edema and dilatation	Crypt degeneration	Denuded villi	Lymphocyte infiltration
Control	0.30±0.48	1.10±0.57	1.40±0.52	0.40±0.52	1.90±0.74	2.10±0.74
Cisplatin	2.20±0.42[a]	2.90±0.57[a]	2.80±0.63[a]	1.70±0.48[a]	2.50±0.71[b]	2.40±0.52[b]
Cisplatin+ Infliximab	3.10±0.74[c,x]	2.10±0.57[c,x]	2.20±0.42[c,x]	1.10±0.57[c,x]	2.20±0.42[d,y]	2.40±0.70[d,y]

[a] Based on the Kruskal-Wallis test there were statistically significant differences between the control group and the cisplatin group in terms of capillary congestion, epithelial sloughing, edema and dilatation, crypt degeneration, denuded villi and lymphocyte infiltration ($p < 0.05$)
[b] Based on the Kruskal-Wallis test there were no statistically significant differences between the control group and the cisplatin group in terms of denuded villi and lymphocyte infiltration ($p > 0.05$)
[c] Based on the Kruskal-Wallis test there were statistically significant differences between the control group and the cisplatin+infiximb group in terms of capillary congestion, epithelial sloughing, edema and dilatation, crypt degeneration, denuded villi and lymphocyte infiltration ($p < 0.05$)
[d] Based on the Kruskal-Wallis test there were no statistically significant differences between the control group and the cisplatin+infiximb group in terms of denuded villi and lymphocyte infiltration ($p > 0.05$)
[x] Based on the Kruskal-Wallis test there were statistically significant differences between the cisplatin group and the cisplatin+infiximb group in terms of capillary congestion, epithelial sloughing, edema and dilatation, crypt degeneration, denuded villi and lymphocyte infiltration ($p < 0.05$)
[y] Based on the Kruskal-Wallis test there were no statistically significant differences between the cisplatin group and the cisplatin+infiximb group in terms of denuded villi and lymphocyte infiltration ($p > 0.05$)

swelling, and cell degeneration were seen. Furthermore, edema was observed in-between crypts. Dense clusters of lymphocytes, plasma cells in groups, eosinophil and neutrophil granulocytes, and edema surrounding the lymphatic and capillaries within the lamina propria were observed. The villi lost their finger-like structure and were oval in shape, their length was reduced due to the effect of edema, and denuded villi were observed. Dense cell swelling and loss as well as degeneration due to vacuolization were observed in columnar cells at the apex, which were prolonged towards the lumen. The goblet cells lost their oval nuclei structures and intense basophilic stain revealed that they were secreting their mucosal content (Figure 1B). Congestion was seen in some areas and edema was surrounding the capillaries. Dense clusters of eosinophil and neutrophil granulocytes were detected between the crypts and near the mucous membranes (Figure 1B- small picture).

The histopathological examination with hematoxylin- eosin staining revealed that there was less tissue and cellular damage in the CPI group when compared to the CP group (Figure 1C). Although the CPI group's crypts were similar to those of the control group in terms of their cylindrical and tightly arrayed structure, their lumens were enlarged. Vacuolization of the crypt epithelium, cell swelling and degeneration were less than was observed in the CP group. Although the lymphocyte density observed in the lamina propria was reduced in the CPI group compared to the CP group, edema and congestion around the lymphatic and capillaries were still observed. The congestion in these groups was observed intensely in the areas close to the ends of the villi. The CPI group's goblet cells had normal morphology, and their basophilic staining was less intense when compared to the CP group. The lengths of the villi in the CPI group had returned to the levels of the control group and the villi regained

Table IV. The immunohistochemical evaluation of the groups.

Groups	Caspase-3	Laminin
Control	2.90±0.57	3.80±0.42
Cisplatin	3.70±0.48 [a]	2.90±0.32 [a]
Cisplatin+Infliximab	3.80±0.42 [b, c]	3.80±0.42 [b, d]

[a] Based on the Kruskal-Wallis test there was statistical significance between the control group and the cisplatin group in terms of caspase-3 and laminin immunopositivity ($p < 0.05$)
[b] Based on the Kruskal-Wallis test there was statistical significance between the control group and the cisplatin+inflixmab group in terms of caspase-3 and laminin immunopositivity ($p < 0.05$).
[c] Based on the Kruskal-Wallis test there was no statistical significance between the cisplatin group and the cisplatin+inflixmab group in terms of caspase-3 immunopositivity ($p > 0.05$).
[d] Based on the Kruskal-Wallis test there was a statistical significance between the cisplatin group and the cisplatin+inflixmab group in terms of laminin immunopositivity ($p < 0.05$).

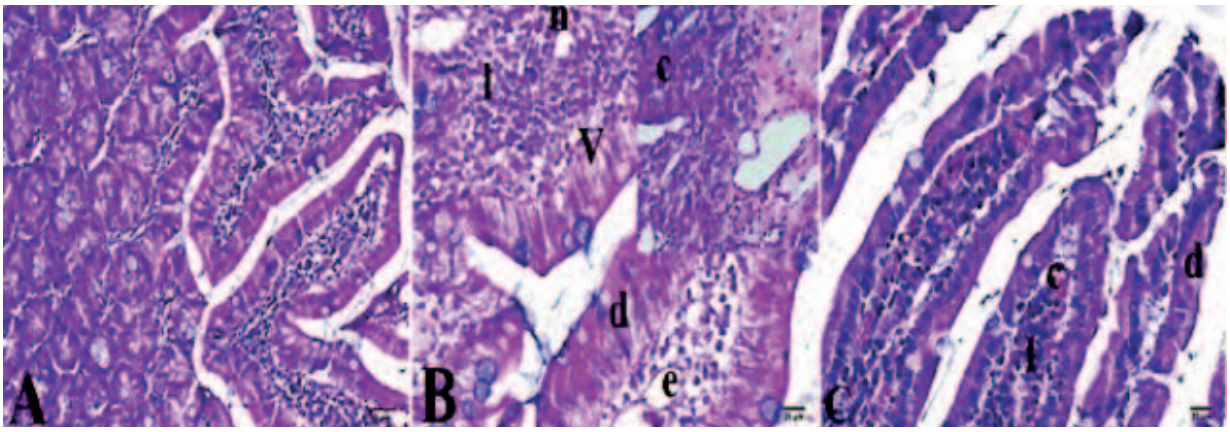

Figure 1. The histopathological examination of the small intestine under the light microscope: **A**, control group; **B**, Cisplatin treated group; c: congestion, d: degeneration, e: edema, V: vacuolization, l: lymphocyte infiltration, n: neutrophils granulocyte clusters; **C**, cisplatin + infliximab treatment group, c: congestion, d: degeneration, l: lymphocyte infiltration, H&E Staining.

their finger-like morphological appearance. In addition, the denudation of the villi structure that is characterized by epithelial swelling and spilling in the surface facing the lumen was less in CPI group than in the CP group (Figure 1C).

Immunohistochemical Data

The anti-Caspase-3 immunopositivity determined by immunohistochemical staining of intestinal tissues with the immunoperoxidase method was 15% (+ +), 55% (+ + +) and 30% (+ + + +) in the C group, 5% (+ +), 30% (+ + +), and 65% (+ + + +) in the CP group and 15% (+ +), 40% (+ + +) and 45% (+ + + +) in the CPI group (Figure 2).

The anti-laminin immunopositivity determined by immunohistochemical staining of intestinal tissues with the immunoperoxidase method was 10% (+ +), 50% (+ + +) and 50% (+ + + +) in the C group, 55% (+ +) , 40% (+ + +) and 5% (+ + + +) in the CP group and 5% (+ +), 35% (+ + +) and 60% (+ + + +) in the CPI group (Figure 3).

Discussion

Chemotherapeutic agents such as CP are also referred to as antineoplastic agents. They are important drugs that are used in cancer therapy and are both more effective and less toxic than other

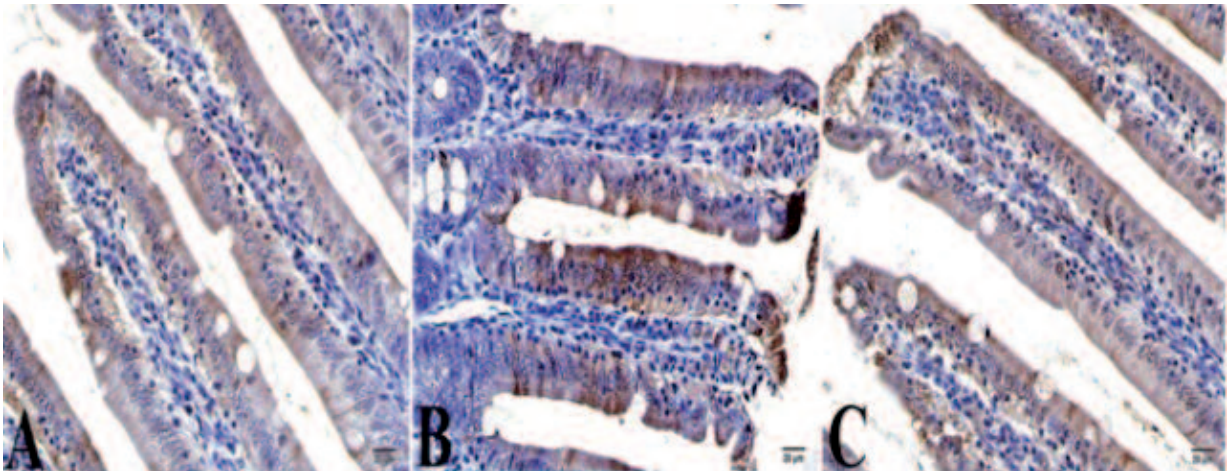

Figure 2. Immunohistochemical staining with the immunoperoxidase method and histopathologic examination of the small intestine under the light microscope. **A**, Control group. **B**, Cisplatin treated group. **C**, Cisplatin + infliximab treated group, immunoperoxidase staining with anti-caspase-3 antibody, H&E Staining.

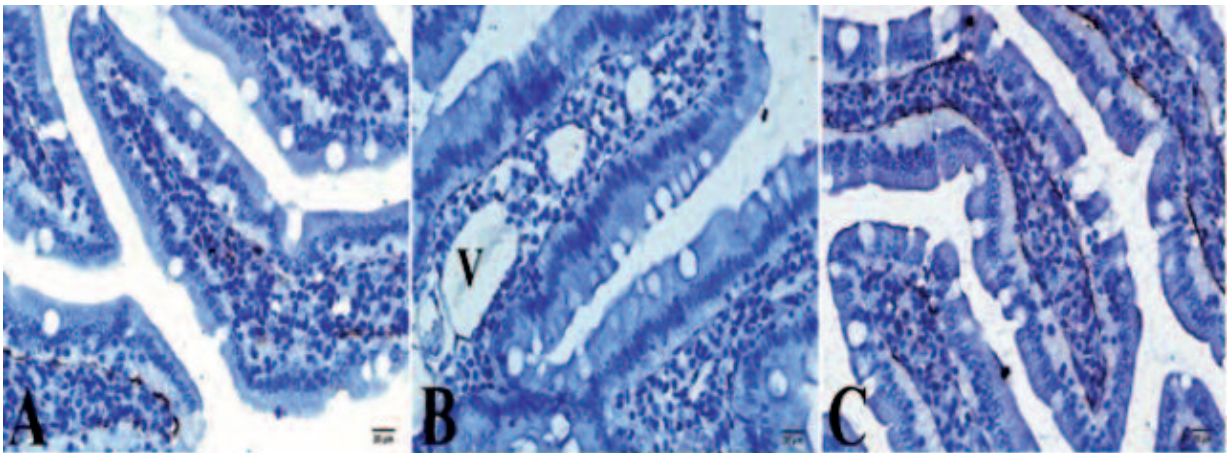

Figure 3. The immunoperoxidase staining and histopathologic examination of the small intestine under the light microscope. **A**, Control group; **B**, Cisplatin treated group; **C**, Cisplatin + infliximab treated group, immunoperoxidase staining with anti-laminin antibody.

chemotherapeutic agents[4]. However, they also have unwanted side effects. Chemotherapeutics may cause oxidative damage in the intestinal tissue and can affect cell structure. This damage may lead to cellular degeneration as well as cell swelling and death. In this study, we aimed to investigate the protective effects of TNF-α blockers (such as infliximab) on the toxicity of chemotherapy drugs by administering infliximab before chemotherapy.

According to some researchers, the most prominent intestinal tissue damage occurs in the epithelial tissue (more specifically, in the apex of the villi and their distal endings)[3]. CP can cause acute epithelial necrosis and apoptosis that leads to damage in the mucosal structure. In the present study, we detected decreased laminin immunoreactivity in the villi apexes, which suggests that the epithelial damage was most prominent in these regions. In addition, damage to the intestinal enterocytes was observed in the form of cytoplasmic vacuolization and multi-nuclei structure. Our findings agree with to those of other studies[3,22].

The gastrointestinal epithelium is an area of tissues with the highest cellular regeneration. CP slows down the tissue regeneration by causing DNA damage at the cellular level, which causes tissue loss[23,24]. In the group treated with CP, we detected degeneration at the villi and at the distal ends of enterocytes. We also observed cytoplasmic vacuoles in the intestinal epithelium as well as subepithelial apoptosis. However, these degenerations were lessened in the intestinal tissues from the CPI group.

Several studies have reported that while CP vastly diminishes cells in the intestinal crypts, it is less effective at the villi[25]. In the CP group, we detected dense cell degeneration and decreased crypt staining, while in the CPI group, there was a decrease in cell degeneration, but there was no change in crypt staining.

Furthermore, we observed dispersion of the goblet cells that were between the intestinal epithelial cells in CP groups. The secretion of mild basophilic mucosal fluid into the lumen was easily detected in these preparations. Goblet cell degeneration and dispersion was particularly observed in the tissues from the CP group, while the damage was decreased in the CPI group.

The heights of the intestinal crypts were similar between the groups. The lamina propria of the villi that face the luminal surface are tissues rich in connective tissue cells. We determined that cellular degeneration and necrosis caused vascular dilatation and an increase in the neutrophil granulocytes in the connective tissue of the intestinal mucosa in rats treated with CP.

CP increases the mitochondrial permeability in the cell via a variety of chemical mechanisms. In our study, the cell loss induced by apoptosis in the group that received CP was shown by caspase 3 immunopositivity, which occurred in the apex of the villi. Immunohistochemical staining showed that there was no significant difference between the CP and CPI groups in terms of caspase 3 immunopositivity ($p > 0.05$). The application of infliximab prior to the injection of CP may reduce the toxic effects of CP and prevent cell and tissue loss.

Various researchers have suggested that CP increases TNF-α values. In our study, we believe that the increased TNFα values in the CP group were caused by the toxicity of CP. However, because there were lower TNF-α values in the CPI group, we believe that the infliximab prevented tissue degeneration and contributed to cell regeneration and prevented apoptosis due to increasing TNF-α values.

Recent studies have shown that infliximab inhibits the functional activity of TNF-α[15]. In our study, the TNF-α values were significantly lower in the CPI group than in the CP group ($p < 0.05$).

Importance of the Present Study

In the literature, we could not find any studies regarding the relationship between laminin and the activity of antineoplastic agents (such as CP). However, laminins were shown to play an important role in the invasion and metastasis of tumour cells[26]. Our studies indicated that there was a significant difference between the three groups in terms of laminin immunopositivity ($p <0.05$). The subepithelial damage generated by CP was shown to be slightly corrected by infliximab. In addition, the protective effect of infliximab on mucosal degeneration and damage to the intercellular connections (generated by CP) and laminin immunopositivity were positively correlated.

In colonic tissue, the epithelial tissues and subepithelial layer are affected the most due to cell degeneration. In our study, immunohistochemical staining showed that laminin immunopositivity was the highest in the CP group, which suggests that the basal membrane and laminin concentration were affected by the toxicity. Apoptosis was decreased in this area in the CP group, which suggests that cell loss might be positively correlated with laminin immune reactivity. We underline that we used adult male Wistar albino rats. However, animal models of small intestine injury may not accurately mimic the human disease process.

Conclusions

In this study, protective effects of infliximab on CP-induced intestinal tissue damage tissues was shown. CP may be used for the treatment of cancer to achieve the less adverse-effects, and that TNF-α blockers such as infliximab should be studied in a human population due to promising results in rats.

Conflict of interest

The Authors declare no conflict of interests.

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