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Beneficial effect of oral administration of zinc sulfate on 5-fluorouracil-induced gastrointestinal mucositis in rats

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Abstract. – OBJECTIVE: This experimental study explored the potential of oral zinc sulfate to protect the gut mucosa from 5-fluorouracil (5-FU)-induced degenerative lesions in Wistar rats.

MATERIALS AND METHODS: Female Wistar rats were used and divided into 2 interventional groups (Z with 6 animals and F with 5 animals) and one control group (M with 5 rats). After 2 hours of fasting, group Z received via oral gavage 1.5 ml of solution, corresponding to 15 mg zinc sulfate for 9 consecutive days. Groups F and M received only the vehicles. On day 3, 400 mg/kg of 5-FU was administered intraperitoneally to groups Z and F. Tissue samples were collected from the duodenum, jejunum, colon and liver. Histological assessment for each gastrointestinal tract segment was determined semi-quantitatively by rating 11 histological features from normal (0) to severe (3). The independent groups were analyzed using the Kruskal-Wallis test and the Mann-Whitney U-test, with a Bonferroni correction for alpha ($p \leq 0.016$).

RESULTS: In group F the jejunum was the most affected area with a mean histological score of 27 (25-32). In the Z group, significantly lower histological scores were obtained compared with group F (duodenum Z *vs.* F: U = 0, $p = 0.004$; jejunum Z *vs.* F: U = 0, $p = 0.006$ and colon: Z *vs.* F: U = 0, $p = 0.005$). Graded liver necro-inflammatory lesions were significantly lower in group Z compared with group F (U = 0, $p = 0.004$), suggesting fewer bacterial intestinal translocation processes.

CONCLUSIONS: Zinc sulfate has a beneficial role, decreasing the severity of gut mucosal injuries induced by 5-FU in Wistar rats.

Key Words:
5-Fluorouracil, Intestinal mucositis, Zinc sulfate.

Introduction

Mucositis is an inflammatory ulcerative disorder of the oral cavity or gastrointestinal (GI) tract, which can be caused by infectious diseases, immune deficiencies or certain drugs, but which is extremely common in patients receiving oncological treatment. The chemotherapy with 5-fluorouracil (5-FU), capecitabine or tegafur can lead to rates as high as 50% for gastrointestinal mucositis[1].

Mucositis is characterized by mucosal breakdown leading to ulceration, allowing bacteria to enter the systemic circulation by translocation[2]. In immunocompromised patients, frequently in those undergoing oncological treatments, this can lead to septic complications and death. In addition, losing the epithelial lining of the gastrointestinal tract, causes impairments in absorption and secretion, with subsequent development of diarrhea, sometimes severe. Histologically, mucositis is characterized by villous atrophy, crypt hypoplasia and dilation, epithelial loss, necrosis, inflammation and excessive mucous secretion[3].

Because of these changes and their corresponding symptoms, patients who develop high grade mucositis require de-escalation of treatment, with delays and hospitalizations that can compromise treatment response and lead to increased mortality rates[4].

The exact mechanisms of oral and GI mucositis are not fully understood. Those principally suggested are alterations in cell absorptive functions, mucin composition and distribution, as well as direct cytotoxic effects from chemotherapeutics (5-FU, irinotecan and methotrexate).

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The subsequent bacterial translocation triggers inflammatory processes[5]. More recently, reported pathogenetic evidence[6-8] from animal and clinical studies revealed disturbances in microbiota in chemotherapy-induced mucositis.

Currently, therapeutic interventions for mucositis are limited, relying on rehydration, correction of electrolyte imbalances and the use of agents that reduce motility or intestinal secretion. Other agents with anti-inflammatory, regenerative or antimicrobial properties that have been tested and proved to be efficient include zinc, palifermin, benzydamine, R-spondin 1 or probiotics[2,9-11].

Zinc is an essential trace element for humans and is required for the function of more than 300 enzymes and 1000 transcription factors[12]. Its roles include supporting the immune system, cell division and regeneration, and protecting against cellular oxidative stress. Zinc has received attention in the past years for its reported efficacy in mucositis given its functions in epithelial proliferation, extracellular matrix synthesis and wound healing[2,13-15]. However, these studies have only experimented with zinc sulfate in treating oral mucositis. We do have evidence[16-18] that indicates the benefit of zinc supplementation in the management of diarrhea, especially in pediatric populations.

In addition, zinc has been proven to interact with bacteria makeup and function, thus being able to indirectly influence the immune system[19,20]. Microbiota communicates with immune cells through a series of toll-like receptors (TLR), such as TLR4. Activation of these receptors leads to stimulation of intracellular signaling pathways and inflammatory cytokine production.

In an experimental model, Kucuk et al[21] documented an increase in acute inflammatory cytokines and endotoxin content with simultaneous bacterial translocation to the portal venous blood, liver, spleen and mesenteric lymph nodes after administration of 5-FU in rats.

This study was designed to assess the potential of zinc sulfate to reduce the necro-inflammatory lesions induced by 5-FU on gut mucosa in Wistar rats.

Materials and Methods

Animal Grouping and Experiment Design

The study was approved by the Animal Ethics Committee of University of Medicine and Pharmacy Cluj-Napoca and by the Romanian National Authority of Veterinary and Food Safety (84/19.07.2017) being in accordance with Romanian laws. The experiments were performed at the Animal Research Laboratories of the Medicine and Pharmacy University in Cluj-Napoca, Romania, based on a previous methodology used by the authors[7]. Female Wistar rats were used and divided into 2 interventional groups (named Z and F) and one control group (M). After 2 hours of fasting, group Z received a zinc sulfate solution *via* oral gavage. The solution was prepared using zinc sulfate heptahydrate powder which was dissolved in distilled water immediately prior to administration. Each animal received 1.5 ml of solution, corresponding to 15 mg zinc sulfate, daily for 9 consecutive days. Groups F and M received only distilled water. Animals were allowed to eat after another 2 hours of fasting. On day 3 of the experiment 400 mg/kg of 5-FU was administered intraperitoneally to groups Z and F. Group M received only sterile saline.

Throughout the experiment, diarrhea was recorded for all animals and graded as follows: 0 - no diarrhea, 1 - mild diarrhea (staining of anus), 2 - moderate diarrhea (staining over the top of the legs and lower abdomen) and 3 - severe diarrhea (staining over the legs and higher abdomen, accompanied by continual oozing)[3].

On day 9, the animals were sacrificed by cervical dislocation.

Collection of Gut and Liver Tissues

Tissue samples were collected from the proximal part of the duodenum, distal part of the jejunum and middle part of the colon, according to Howarth et al[22]. In addition, liver (left lateral lobe and right medial lobe) samples were harvested for histological examination, as described by Ruehl-Fehlert et al[23].

Histological Examination and Assessment

For the histological examination, the tissues were fixed in 10% phosphate-buffered formalin for 24 hours, routinely processed and embedded in paraffin wax. For histological examination, transverse sections of 3 μm were stained with hematoxylin and eosin (H&E). The sections were independently examined by two pathologists (M. Taulescu and C. Toma) using an Olympus BX-41 microscope. The photomicrographs were taken using an Olympus SP 350 digital camera and

processed using Stream Basic imaging software (Olympus Corporation, Tokyo, Japan). When there was a divergence of opinion, an agreed upon diagnosis was reached through simultaneous evaluation in a multi-head Zeiss Axio Scope A1 microscope.

The degree of mucositis was evaluated using a method previously described by Howarth et al[24]. Semi-quantitative histological assessments for each intestinal segment (duodenum, jejunum, and colon) were obtained by rating histological characteristics of mucositis from normal (0) to severe (3). The employed criteria included: villous fusion and stunting (atrophy), disruption of brush border and surface enterocytes, reduction in the number of goblet cells, reduction in numbers of mitotic figures, crypt loss/architectural disruption, disruption or distortion of crypt cells, crypt abscess formation, the presence of polymorphonuclear cells or lymphocytes, dilatation of lymphatic vessels and capillaries, thickening and edema of the submucosa and/or muscularis externa. Histological changes of the hepatic parenchyma were graded according to previous studies[25].

For the immunohistochemical method the sections were incubated at 37°C for 12 hours and were processed using the automatic platform Leica BOND-MAX. The primary mouse-monoclonal antibody anti-TLR4, (ab22048, Abcam, Cambridge, UK) was diluted in 1% PBS-BSA (bovine serum albumin) at 1:100. The Bond Polymer Refine Detection kit (DS9800, Novocastra, Wetzlar, Germany) containing peroxide block, post-primary, polymer reagent, DAB chromogen and hematoxylin counterstain were used. The negative controls for each sample were prepared by replacing the primary antibody with mouse IgG1 Negative Control (Code X0931, Dako, Glostrup, Denmark). Immunopositivity for TLR4 was evaluated by a pathologist who separately assessed the mucosal epithelium, glandular epithelium, and lamina propria[26]. The assessment was microscopically graded as follows: 0 – the same as background, 0.5 – close to background, 1 – well marked positivity, 1.5 focally enhanced, 2 – strong positivity, 2.5 – very strong positivity[27].

Statistical Analysis

For the statistical analysis, IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA) was used. The histological and immunohistochemistry scoring results were expressed as median interquartile ranges (IQR). The independent groups were analyzed using the Kruskal-Wallis, Mann-Whitney *U* test. If the Kruskal-Wallis test led to significant results, a Bonferroni correction for alpha was applied on the Mann-Whitney U test, with a p-value ≤ 0.016 being considered statistically significant.

Results

Sixteen adult female Wistar rats with an average weight of 300 g were used for the experiment. The animals were divided into 3 groups: group M and F which consisted of 5 animals, and group Z which included 6 animals. 5-FU induced diarrhea in all rats in both groups where it was administered. However, the diarrhea scores were significantly lower in group Z than in group F ($p < 0.05$) with later onset (Table I).

No significant intestinal and hepatic histological changes were found in the control group (Figure 1 A1-A4).

In group F, which underwent intraperitoneal administration of 5-FU, the jejunum was the most affected area, with the duodenum closely following. Mean histological severity scores were 21 (range 19-30) for the duodenum and 27 (range 25-32) for the jejunum (Table II). The most important lesions in the small bowel were villous atrophy, disruption of brush border and surface enterocytes, epithelial necrosis, crypt loss with architectural disruption, crypt abscess formation, edema, hemorrhages and infiltration of lamina propria with large numbers of neutro-

Table I. Diarrhea score in rats across the experiment.

Group	Diarrhea score									Average score
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	
M	0	0	0	0	0	0	0	0	0	0
F	0	0	0	1	1	1	1	2	2	0.89 ± 0.78
Z	0	0	0	0	0	0	1	1	1	0.33 ± 0.5

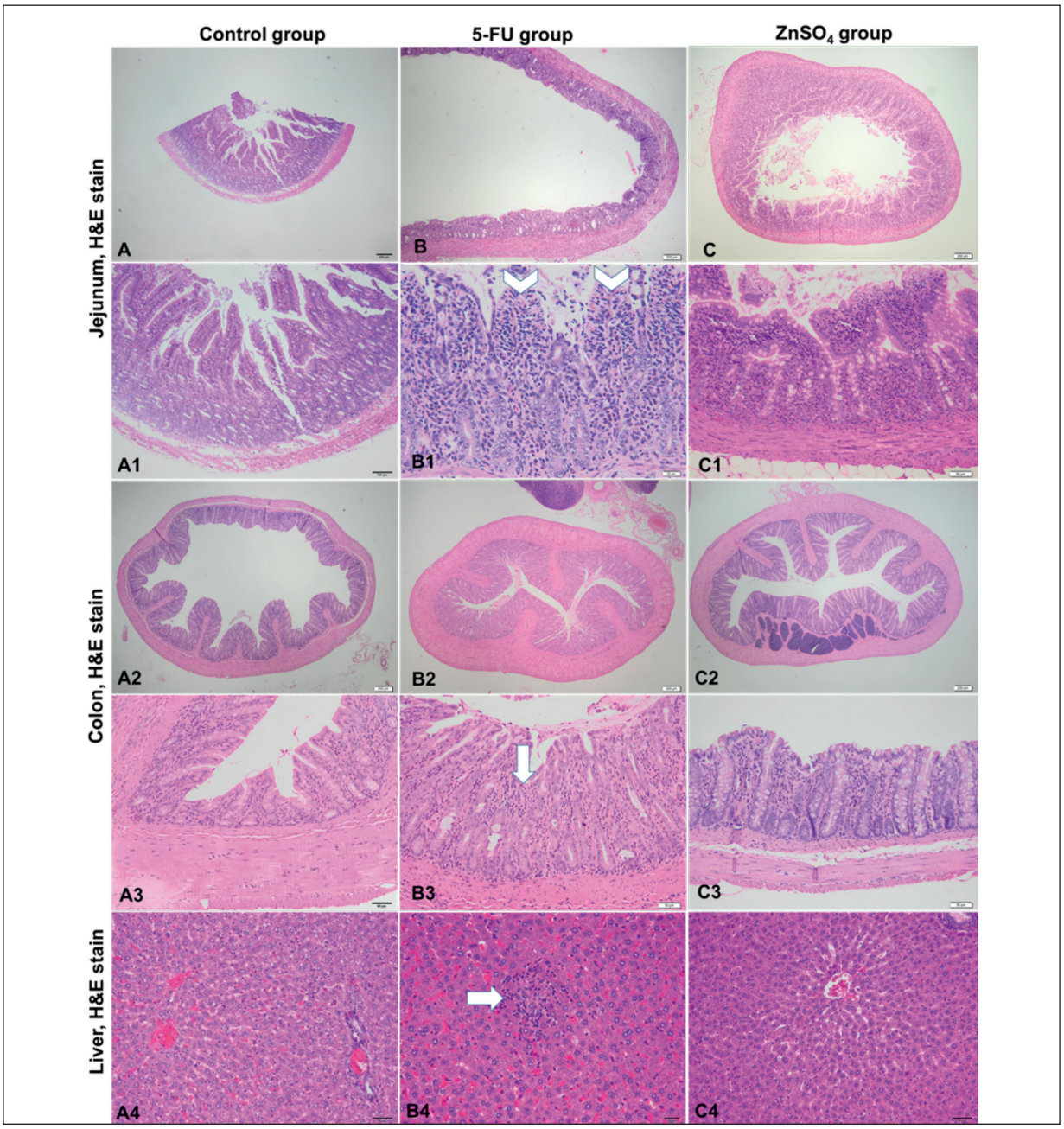

Figure 1. Microscopical findings of the intestinal mucosa from experimental Wistar rats exposed to 5-FU-induced intestinal mucositis. **Control group:** The photomicrographs of the intestinal mucosa and liver showing no significant histological changes: A and A1) jejunum, A2 and A3) colon, and A4) liver. HE stains, Bar=200 μm, 100 μm, 200 μm, 50 μm, 50 μm. **F group:** Significant histological changes of the jejunal (B and B1) and colonic (B2 and B3) mucosa (*white arrows*). Focal necrotizing hepatitis (*arrow*). HE stains, Bar=200 μm, 20 μm, 200 μm, 50 μm, 20 μm. **Z group:** Photomicrographs of jejunal mucosa (C and C1) showing moderate villous atrophy and inflammatory activity, and colonic mucosa (C2 and C3) with minimal inflammatory changes. No significant histopathological changes of the hepatic parenchyma are observed. HE stains, Bar=20 μm, 50 μm, 200 μm, 50 μm, 50 μm. (Abbreviations: 5-FU: 5-fluorouracil; ZnSO4: zinc sulfate).

phils, lymphocytes and fewer macrophages and eosinophils. A few epithelial cell divisions were identified at this level (Figure 1 B and B1). The colonic mucosa was less affected, with a mean histological severity score of 5 (range 3-10) (Table II). Infiltration with polymorphonuclear cells and lymphocytes, as well as mild degenerative changes of the colonic epithelium were depicted

Table II. Descriptive statistics concerning microscopic degenerative lesions, TLR immune staining between interventional groups.

Site	Parameters	Group	Min	Max	Percentiles		
					25th	Median	75th
Duodenum	Histological score	F	19	30	19.5	21	27
		Z	0	18	0.75	1.5	13.5
	TLR4 surface epithelium	F	0.5	2.5	0.625	1	2.125
		Z	0.5	1	0.5	0.5	0.625
	TLR4 glandular epithelium	F	0.5	1	0.625	1	1
		Z	0.5	1	0.5	0.5	0.625
	TLR4 lamina propria	F	0.5	1.5	0.5	1	1.5
		Z	0	0.5	0	0	0.5
Jejunum	Histological score	F	25	32	25.5	27	30
		Z	1	14	1	7	14
	TLR4 surface epithelium	F	1	2.5	1	1.5	2.375
		Z	0.5	1	0.5	1	1
	TLR4 glandular epithelium	F	0.5	2	0.625	1.25	1.875
		Z	0.5	1	0.5	1	1
	TLR4 lamina propria	F	1	2	1.125	1.5	1.875
		Z	0.5	1	0.5	0.5	0.625
Colon	Histological score	F	3	10	3.5	5	7.5
		Z	0	1	0	0.5	1
	TLR4 surface epithelium	F	0.5	1	0.5	0.75	1
		Z	0	1	0	0.25	0.625
	TLR4 glandular epithelium	F	0.5	0.5	0.5	0.5	0.5
		Z	0	1	0	0.5	1
	TLR4 lamina propria	F	0.5	0.5	0.5	0.5	0.5
		Z	0	0.5	0	0.25	0.5
Liver	Histological score	F	2	5	2.5	3	4
		Z	0	1	0	0.5	1

Zinc plays a role in maintaining the integrity of the intestinal barrier function.

(Figure 1 B2 and B3). The liver parenchyma was moderately congested and showed multifocal and mild periportal mononuclear infiltration. Small foci of periportal and intralobular coagulative necrosis associated with mixed inflammatory infiltrates were also observed (Figure 1 B4).

In group Z, the histological changes of the intestinal mucosa were less severe than in group F, and the jejunum showed the highest mean histological severity scores, with a value of 7 (range 1-14). The duodenum showed mean scores of 1.5 (range 0-18) while the colon was normal in aspect, with a mean score of 0.5 (Table II). The most important histological changes identified in the duodenum and jejunum were the presence of polymorphonuclear cells and lymphocytes in the lamina propria, with villous atrophy and a reduction in numbers of mitotic figures being present in varying degrees in some animals. The epithelial injuries were minimal or absent in some individuals (Figure 1 C and C1). Minimal changes were identified in the colonic mucosa, represented by mild infiltration of the lamina propria with neutrophils and lymphocytes (Figure 1 C2 and C3). The liver showed scattered inflammatory cells in the portal spaces, without features of hepatocellular necrosis (Figure 1 C4).

Statistical analysis of graded microscopic degenerative lesions of the duodenum depicted significant differences between groups M *vs.* F ($U = 0$, $p = 0.005$, Mann-Whitney U test) and F *vs.* Z ($U = 0$, $p = 0.004$, Mann-Whitney U test) with no difference between groups M *vs.* Z ($U = 10$, $p = 0.29$, Mann-Whitney U test). Graded microscopic degenerative lesions of the jejunum were significantly different between groups M *vs.* F ($U = 0$, $p = 0.005$, Mann-Whitney U test), F *vs.* Z ($U = 0$, $p = 0.006$, Mann-Whitney U test), but not significant between M *vs.* Z ($U = 5$, $p = 0.034$, Mann-Whitney U test). Also, the colon statistical analysis of graded microscopic degenerative lesions was significantly different between groups M *vs.* F ($U = 0$, $p = 0.005$, Mann-Whitney U test), F *vs.* Z ($U = 0$, $p = 0.005$, Mann-Whitney U test), with no difference between groups M *vs.* Z ($U = 10$, $p = 0.174$, Mann-Whitney U test).

Graded liver necro-inflammatory lesions were significantly higher in group F compared with groups Z and M [M *vs.* F (U = 0, p = 0.008, Mann-Whitney U test), F *vs.* Z (U = 0, p = 0.004, Mann-Whitney U test, M *vs.* Z (U = 10.5, p = 0.429, Mann-Whitney U test)].

Regarding TLR4 staining, group Z showed lower positivity in the mucosal epithelium, glandular epithelium and lamina propria compared to group F in the jejunum, duodenum and colon (Table I). However, when comparing groups, there was no significant difference between them regarding the mucosal or glandular epithelium in the duodenum, jejunum or colon. The only notable significant difference was identified when comparing the jejunal lamina propria TLR4 staining between groups F *vs.* Z (U = 5, p = 0.008, Mann-Whitney U test).

Discussion

Zinc has been recently rediscovered as an essential biological cofactor. Its stable form (Zn^{2+}) has been found to modulate the cellular signal reception, the second messenger metabolism, the protein kinase and protein phosphatase activities, as well as the DNA binding of transcription factors, with new evidence suggesting that it also functions as a modulator of cell proliferation, differentiation and death[28].

One of the fundamental biological processes in which zinc is implicated is apoptosis. Disease or exposure to toxins may lead to this mode of death, by way of zinc deprivation or zinc chelation. Zinc depletion triggers caspase activation leading to apoptosis. However, some authors suggest that zinc does not directly regulate apoptosis, but rather exerts a cytoprotective effect, and when its levels are reduced, cells are exposed to harmful agents that can lead to apoptosis or necrosis[29]. Another way through which zinc can become cytotoxic is if its extracellular concentration exceeds the capacity of the zinc homeostatic system. In this case, the higher concentrations of extracellular zinc result in the destruction of the plasma membrane[30].

Zinc also plays a role in maintaining the integrity of the intestinal barrier[31]. Its depletion leads to reduced expressions of several proteins, resulting in decreased tight junctions in Caco-2 cells[32]. If this happens, water, transported solutes, as well as bacteria can leak through paracellular spaces and into the systemic circulation. Constituting another biological barrier, intestinal Paneth cells hold zinc granules which, together with various antimicrobial agents, are secreted when bacteria bind to intestinal receptors, such as TLR.

Zinc deficiency may occur either as a result of reduced dietary zinc intake, or in the context of an inflammatory gastrointestinal disorder. In the latter case, absorption may be affected secondary to destruction of the digestive tract mucosa, eventually leading to zinc deficiency. Examples of such disorders include gastrointestinal infectious diseases, inflammatory bowel diseases or chemo- or radiation-induced mucositis.

Chemotherapy induces mucositis by killing progenitor cells in the crypts of Lieberkühn and the bases of villi, as well as through apoptosis[33]. The first effects of chemotherapy are the generation of reactive oxygen species and the activation of the transcription factor nuclear factor kappa B (NF-κB) leading to the upregulation of pro-inflammatory cytokines. Subsequently, the tumor necrosis factor (TNF)-α is also activated. Finally, several positive feedback in NF-κB and TNF-α loops occur, leading to inflammation, ulceration and bacterial colonization, through involvement of B-cell lymphoma 2 (Bcl-2), p53 and caspases[34]. All these processes lead to breakdowns in the intestinal barrier.

Based on these arguments, some authors have tested the effectiveness of various zinc preparations speculating that they will be of benefit to prevent or treat several gastrointestinal disorders. Polaprezinc, a chelated form of zinc and L-carnosine, is used in Japan to treat gastric ulcers[35]. This drug increases the expression of various antioxidant enzymes and inhibits the activity of NF-κB and decreases the expression of various inflammatory cytokines, including interleukin (IL) 1beta, IL-6, IL-8, and TNF-α. Recently, Kobayashi et al[36] detected that a high intake of zinc has a protective effect on the development of ulcerative colitis. Furthermore, other authors[37-39] reported that polaprezinc was a useful therapeutic agent for the treatment of small intestinal injury and colitis.

Zinc sulfate might provide clinical benefits in preventing or reducing incidence, severity, or pain intensity of chemotherapy-induced oral mucositis in cancer patients. However, the reported studies[13-15,40,41] have conflicting results with significant biases. Mehdipour et al[15] tested a zinc sulfate mouthwash in patients undergoing chemotherapy for acute leukemia and found a significant decrease in oral mucositis severity in the intervention group of patients receiving zinc sulfate

mouthwash at 4 and 6 weeks (p=0.025). Similarly, Arbabi-kalati et al[14] administered 220 mg zinc sulfate capsules in chemotherapy patients with the control group receiving placebo capsules. In the zinc sulfate group, the occurrence of grade 3 oral mucositis was lower, with patients reporting less xerostomia and pain[14]. Other studies[40,41] tested the efficacy of zinc sulfate capsules in patients undergoing chemotherapy for leukemia and found that it reduced the severity of oral mucositis in these patients. However, the patients from all these reported studies had undergone normal dose chemotherapy. When assessing the effect of zinc on oral mucositis in patients assigned to high-dose chemotherapy, Mansouri et al[13] found that its administration does not have any clinical benefits in the prevention or reduction of severity, and duration of mucositis.

Zinc sulfate is one of the zinc inorganic salts, alongside zinc gluconate or zinc acetate, and can be used as a dietary supplement in animals or humans. Based on the available literature, individual requirement for zinc range from 7.5 to 12.7 mg/day for women and from 9.4 to 16.3 mg/day for men[42]. The pharmaceutical preparations available on the market have a variable concentration of zinc ranging from 15 to 50 mg. For our study we used a solution of zinc heptahydrate dissolved in distilled water so that each animal received 15 mg of zinc daily, corresponding to about 50 mg/kg. Higher doses of zinc have been reported to cause nausea, vomiting and ataxia. We have not encountered any symptoms in our animal model.

5-FU is a pyrimidine analogue which acts in several ways, but principally as a thymidylate synthase inhibitor. Inhibition of this enzyme blocks the synthesis of thymidine, which is required for DNA replication. As a result of this impairment, cells undergo death. Unfortunately, not only cancer cells are affected by 5-FU, but also rapidly proliferating crypt stem cells. Although 5-FU-associated intestinal mucositis has not been fully understood yet, it is reasonable to believe that it is a result of various events, such as inflammation, apoptosis and impairment of crypt cell proliferation. After administration of 5-FU, gastrointestinal mucositis is most evident in the small bowel, but it also occurs in the colon. These changes appear within 1 week of therapy. Our experimental model depicted severe histological lesions in the duodenum and jejunum of animals exposed only to 5-FU, while mild lesions were encountered in the colon. In comparison, in group Z, the injuries of the small intestine were significantly milder, and the large intestine had a microscopic appearance close to normal, with no significant difference between groups M and Z.

These data suggest that oral administration of zinc might have a beneficial effect on 5-FU-induced lesions. In our study we started zinc administration 48 hours before intraperitoneal administration of 5-FU in order to correct possible pre-existing dietary deficits. For this reason, we cannot say whether oral supplementation with zinc could also have a prophylactic, not just therapeutic, effect on gastrointestinal mucositis.

The most likely protective effect of zinc against 5-FU induced mucositis is maintaining the integrity of the intestinal barrier. This hypothesis might also explain the faint TLR4 immunopositivity observed in group Z, even in the absence of statistical significance. Because the intestinal barrier was maintained, less TLR receptors were activated by bacterial translocation. Another argument for the maintenance of intestinal barrier integrity is the lack of liver necro-inflammatory lesions in group Z compared with group F.

Even though zinc sulfate appears to have a beneficial effect in reducing the 5-FU-induced mucositis, the pathogenic mechanisms were still unknown. Further researches should evaluate the influence of zinc preparations on 5-FU efficiency, especially when it comes to digestive tumors.

The novelty of this investigation is the use of zinc sulfate in an experimental model of intestinal 5-FU-induced mucositis. Previous studies have demonstrated that oral administration of another type of zinc, polaprezinc (a chelated form of zinc and L-carnosine) attenuated 5-FU induced intestinal mucositis in a mouse model[43] and was a therapeutic agent for the treatment of small intestinal injury and colitis, caused by acetylsalicylic acid[39]. The protective effects of zinc sulfate were only investigated on oral chemotherapy induced mucositis[14,15,40,41].

The limitations of our study are the reduced sample sizes and the lack of different doses of zinc sulfate analysis. In addition, our study could have benefitted from an analysis of the intestinal microbiota implicated in gastrointestinal mucositis and its relationship to zinc supplementation.

Conclusions

In this animal model study, we demonstrated that zinc sulfate has a beneficial role on the intestinal mucositis secondary to 5-FU admin-

istration. To the best of our knowledge, this was the first experimental research investigating the effects on oral zinc sulfate on 5-FU-induced intestinal mucositis. Further studies are required to detect the optimal dose and its potential protective role in humans.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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